Inhibition of Class I HDAC Ameliorates Acute and Chronic Pancreatitis by Reducing Leukocyte Recruitment, Acinar-to-Ductal Metaplasia and Fibrosis

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Summary

1.1 Zusammenfassung

Pankreatitis ist eine entzündliche Erkrankung des Pankreas. Es wird durch verfrühte Aktivierung der Verdauungsenzyme in den Azinuszellen verursacht, was zu einer Selbstverdauung führt. Die milden Formen der Pankreatitis heilen normalerweise von selbst ab. Im Gegensatz dazu, sind die schweren Formen durch Nekrosen, Infektion, Organversagen und hohe Mortalität gekennzeichnet. Bei der chronischen Pankreatitis geht eine persistierende Entzündung mit Organschädigung einher. Dies führt zu intestinaler Maladsorption und endokrinem Funktionsverlust. Deshalb ist Pankreatitis immer noch eine klinische Herausforderung, assoziiert mit hoher Morbidität und Mortalität. Sowohl die akute wie auch die chronische Pankreatitis zeigen in vielen Ländern eine steigende Inzidenz. Es gibt keine kurativen Therapien, nur systemische Behandlungen mit unterstützender Wirkung. Es besteht also ein dringender Bedarf nach spezifischen Therapieansätzen, die aufgrund von Forschungserkenntnissen etabliert werden können.

Epigenetische Modifikationen regulieren die Transkription der Gene, ohne eine Veränderung der zugrundeliegenden DNA-Sequenzen. Die Acetylierung ist eine der bekanntesten Modifikationen, die bei der Kontrolle der Transkriptionsaktivität von Histon-Proteinen ansetzt. Dabei spielen die Histonacetylasen (HAT) und Histon-Deacetylasen (HDAC) bei der Histon-Modifikation eine wesentliche Rolle, Histone werden acetyliert oder deacetylier. Die HATs spielen dabei eher die Rolle der Gen-Aktivatoren während die HDACs die Gen-Inaktivierung fördern. Es ist jedoch so, dass HDAC auch einzelne Gene selektiv aktivieren können, was die Komplexität der Genregulation durch HDACs unterstreicht. In der Pathophysiologie vieler entzündlicher Erkrankungen scheint die Expression und Aktivität der HDACs eine bedeutende Rolle zu spielen, es ist jedoch nicht bekannt, ob HDACs auch bei der Pankreatitis wesentlich den Verlauf beeinflussen. In dieser Studie wurde deshalb untersucht, ob durch HDAC regulierte epigenetische Mechanismen in verschiedenen pankreatischen Krankheiten aktiv sind. Es wurden dazu Mausmodelle von akuter, chronischer und autoimmuner Pankreatitis verwendet. Wir konnten zeigen, dass während der entzündlichen Erkrankung die HDAC sowohl durch Transkription als auch Enzymaktivität hoch-reguliert wurden. Die grösste Veränderung wurde bei den Klasse I HDAC Enzymen beobachtet. Parallel dazu wurde eine Reduktion der Acetylierung von Kernproteinen festgestellt, was mit der Erhöhung der HDAC-Aktivität erklärbar ist. Um die Aktivität der Klasse I HDAC weiter zu charakterisieren, wurde ein selektiver Inhibitor (MS-275) verwendet, der sowohl in der experimentellen Pankreatitis, wie auch in Zellkultur an Makrophagen und Zell-Explantaten getestet wurde. Der Inhibitor hatte keinen Einfluss auf den primären Zellschaden, reduzierte jedoch die entzündliche Reaktion, sowohl der Makrophagen wie auch der T-Zellen während akuter und chronischer Phase. Zusätzlich untersuchten wir den Effekt der HDACs auf die regenerative Phase. Bei der Pankreasregeneration können Azinuszellen direkt proliferieren oder es findet eine Dedifferentiation der azinären Zellen statt (Azinus-zu-Dukt Metaplasie, ADM). Dieser Zustand gleicht einer Vorgängerzelle, die sich auch teilen kann. Diese Untersuchungen wurden wiederum am Tiermodell, sowie an Zellkulturen (AR42J-Zellen) mittels des spezifischen Klasse I HDAC Inhibitors MS-275 vorgenommen. Der Inhibitor induzierte die Transkription von Zyklinen und reduzierte damit die Proliferation der Azinuszellen. Zudem reduzierte MS-275 den DNA-Schaden und limitierte die Dedifferentiation in ADM, was sich auch auf die SOX9 Genexpression auswirkte. Schliesslich haben wir auch die Rolle der HDACs

in der Fibrose untersucht und konnten feststellen, dass die Inhibition mittels MS-275 zu einer Reduktion der Fibrose als Folge einer Interferenz des TGFβ-Signaltransduktionsweges führte.

1.2 Signifikanz des Projektes

Unsere Untersuchungen an Mausmodellen und *in vitro* Zellversuchen haben gezeigt, dass die epigenetische Regulation der Acetylierung durch die Klasse I HDACs eine wesentliche Rolle in der Pathophysiologie der akuten und chronischen Pankreatitis spielt. Vor allem zeichnet sich ein direkter Einfluss dieser Enzyme auf die entzündliche Reaktion, die Proliferation und Dedifferentierung der Azinuszellen, sowie des fibrotischen Prozesses ab. Zusammenfassend unterstützen unsere Daten ein mögliches Einsetzen von HDAC Inhibitoren als therapeutische Strategie zur Behandlung von Pankreatitis. Des Weiteren würde sich die beobachtete Hochregulation der Klasse I HDACs während der Autoimmun Pankreatitis als mögliches Ziel einer therapeutischen Intervention bei dieser Krankheit anbieten, insbesondere, weil die heutige Therapie mit vielen Rückfällen konfrontiert ist.

1.3 Summary

Pancreatitis is a disease characterized by pancreatic inflammation. It is caused by premature intra-pancreatic activation of digestive enzymes stored in acinar cells, leading to auto-digestion of the organ. Mild forms of acute pancreatitis are in general self-limiting. On the contrary, severe forms of the disease are associated with pancreatic necrosis, infection, organ failure and high mortality rates. Chronic pancreatitis is a long-term inflammation where the pancreas becomes permanently damaged, leading to intestinal malabsorption and endocrine dysfunction. Pancreatitis is still a clinical challenge associated with high risk of severe morbidity and mortality. Both acute and chronic forms of pancreatitis have rising incidence in many countries. There is currently no cure for pancreatitis and the treatment is limited to supportive therapy. Thus, there is an urgent need for new therapeutic interventions. For this reason, further investigations are required to identify and develop new treatments for this debilitating disease.

Epigenetic modifications regulate gene transcription without changing the DNA sequence. Acetylation is one of the most common epigenetic modification of histones and plays a major role in gene transcription control. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are two families of enzymes that catalyze acetylation and deacetylation of histones, respectively. HATs are regarded as activators and HDACs as repressors of gene expression. Nevertheless, HDACs have also been linked to transcriptional activation of selected genes and deletion or inhibition of HDACs often results in the up-regulation or down-regulation of similar numbers of genes, indicating the complexity of HDAC-mediated control of gene transcription. HDACs expression and activity appear to contribute to the pathology of many inflammatory diseases. However, the role of HDACs during pancreatitis has not yet been investigated. Thus, in this study, we evaluated whether epigenetic mechanisms regulated by the activity of HDACs are involved in the development of cerulein-induced acute, chronic and autoimmune pancreatitis using different mouse models. We demonstrated that HDACs' expression and activity were up-regulated in a time-dependent manner during the course of pancreatic inflammation, with the highest abundance observed for class I HDACs. Concomitant to this, decreased acetylation of nuclear proteins was observed. The functional relevance of class I HDACs was tested with the selective class I HDAC inhibitor MS-275, both *in vivo* upon cerulein induced-pancreatitis and *in vitro* in activated macrophages and primary acinar cell explants. MS-275 treatment did not prevent the initial acinar cell damage. However, it effectively reduced the infiltration of inflammatory cells, including macrophages and T cells, in both acute and chronic phases of the disease, and directly perturbed macrophage activation. In addition, we analyzed whether HDACs regulate the regenerative process of the pancreas following pancreatitis. Pancreatic regeneration involves proliferation of mature acinar cells and a transient de-differentiation of acinar cells to acinar-to-ductal metaplasia (ADM), a progenitor-like state, followed by cell division and re-differentiation. HDAC-dependent regulation of pancreatic regeneration was investigated *in vivo* and *in vitro* in the acinar cell line AR42J and in primary acinar cells with the specific class I HDAC inhibitor MS-275. Pharmacological inhibition of class I HDAC down-regulated the expression of late cyclins and reduced the proliferation of acinar cells. In addition, MS-275 treatment reduced DNA damage in acinar cells, down-regulated the progenitor-like gene Sox9 and limited acinar de-differentiation into ADM in a cell-autonomous manner by disrupting the EGFR signaling axis. Finally,

we analyzed the effect of MS-275 upon induction of chronic pancreatitis. We found that the inhibitor reduced the development of fibrosis by down-regulating TGF-β signaling in the pancreas.

1.4 Significance of the project.

Our *in vivo* and *in vitro* investigations demonstrate that epigenetic regulation of acetylation mediated by class I HDACs plays a crucial role in the pathophysiology of acute and chronic phases of pancreatitis. Specifically, this study underlines the direct involvement of these enzymes in the development of inflammatory response, in the proliferation and de-differentiation of acinar cells into metaplastic lesions and in the fibrotic process in the damaged pancreas. Collectively, our data support the potential of therapeutic strategies based on inhibition of class I HDACs during acute and chronic inflammatory insults of the pancreas. In addition, the upregulation of HDAC isoforms observed during autoimmune pancreatitis prompts the exploration of the therapeutic effect of HDAC inhibition in this type of chronic pancreatitis, the treatment of which remains challenging due to the high frequency of relapses.

Introduction

2.1 Pancreas anatomy and physiology.

The pancreas is an endocrine and exocrine gland of the gastrointestinal system. Anatomically, the pancreas is divided into three parts: the head that lies near the duodenum, the body, which includes the majority of the gland, and the tail that ends at the hilum of the spleen. Histologically, the endocrine and exocrine pancreatic tissue constitute 2% and 95% of the total pancreatic mass, respectively (Figure 1A).

The endocrine tissue consists of different kinds of cells organized into islets of Langerhans distributed along the organ. Each type of cell synthetizes specific hormones like insulin, glucagon, somatostatin, and pancreatic polypeptide and, following an appropriate stimulus, the hormones are secreted into the bloodstream to control blood sugar, energy metabolism, and storage throughout the body (Figure 1B).

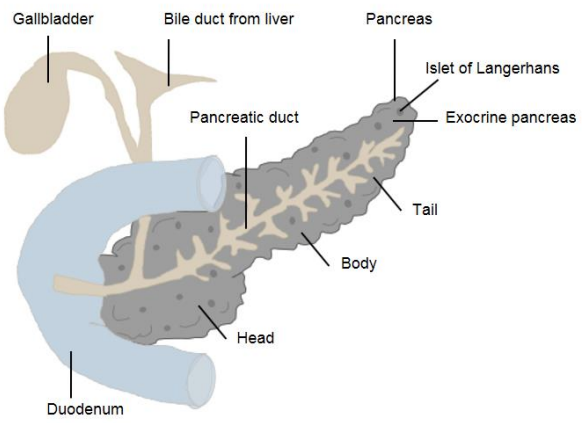
The exocrine tissue includes acinar cells organized in acini and ductal cells with associated connective tissue, vessels and nerves. The exocrine pancreas plays a central and essential role in the digestion of proteins, carbohydrates, and fat. (Figure 1B).

2.1.1 The function of pancreatic acinar cells.

Acinar cells are specialized to synthesize, store, and secrete different digestive enzymes. Although pancreatic amylase and lipase are secreted as active enzymes, generally the pancreatic digestive enzymes such as proteases and several other hydrolases are secreted as inactive zymogens [1]. Digestive enzymes and zymogens are secreted via the pancreatic duct into the duodenum where they are required for the digestion progression. There, these zymogens are converted into a mature and active enzymatic form by proteolysis, cleaving off a pro-peptide. Pancreatic secretion consists of an enzymatic component from acinar cells and an aqueous bicarbonate solution from the duct cells. Pancreatic secretory response is regulated by both neural and endocrine stimulation and their respective specific neurotransmitters and hormones receptors. Both kinds of receptors are present on the basolateral membrane of acinar cells. Furthermore, like the stomach, the pancreas is innervated by the vagus nerve that activates intra-pancreatic postganglionic neurons releasing acetylcholine (Ach), the primary secretagogue for acinar secretion, which initiates a modest secretory stimulus in response to ingestion of a meal. Acinar secretion is strongly stimulated upon food entry into the stomach by the hormones secretin and cholecystokinin (CCK), produced by intestinal endocrine cells upon detection of chyme. Both secretin and CCK stimulate acinar cells to produce and secrete their zymogen content, while inducing the gallbladder to contract and release bile into the duodenum [2]. In addition, secretin stimulates pancreatic and biliary ducts to secrete into the lumen bicarbonate, which neutralizes the acids, thereby balancing the pH and preventing acid-induced damage of the organs. (Figure 1C).

Figure 1.

A



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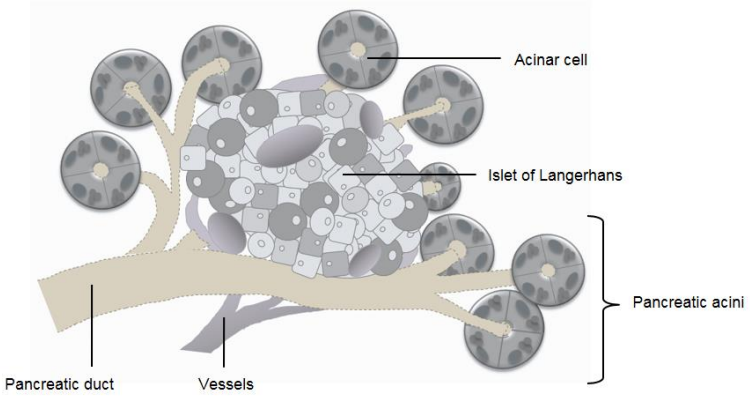

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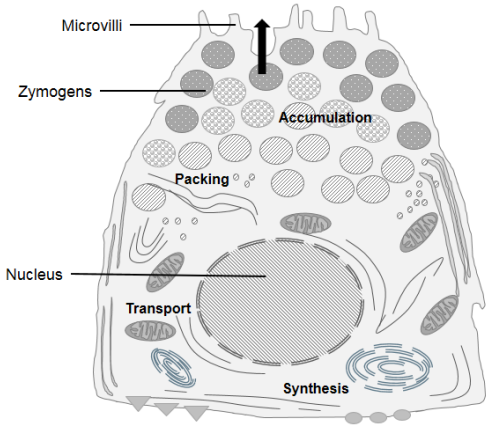

Figure 1A. General anatomy of the pancreas. **Figure 1B.** A pancreatic islet of Langerhans embedded in exocrine tissue. **Figure 1C.** A single acinar cell. Black arrow indicated where zymogens are secreted.

2.2 Pathophysiology of pancreatitis.

2.2.1 The inflamed exocrine pancreas: acute, chronic and autoimmune pancreatitis.

Pancreatitis is an inflammation of the pancreas characterized clinically by abdominal pain, nausea and elevated levels of pancreatic enzymes, including amylase and lipase, in circulation. Acute pancreatitis is one of the most common diseases in gastroenterology. Repeated events of acute pancreatitis can lead to chronic pancreatitis. Both forms are serious and may result in severe complications. Progression from acute pancreatitis with mild symptoms to severe pancreatitis is a clinical challenge associated with high a morbidity and mortality rate. Although most of pancreatitis cases are mild and can be resolved within days or weeks, more severe cases are lethal due to multi-organ failure. The incidence of acute pancreatitis varies between populations from 4,9 to 35 per 100,000/year with considerable morbidity and mortality rates. Chronic pancreatitis also has a high mortality rate. However, it is variable depending on the cultural background. Nonetheless, acute and chronic pancreatitis consistently affect males twice as often as females. The etiology and pathogenesis of pancreatitis has been thoroughly investigated worldwide. Multiple factors can initiate or enhance injury of pancreatic cells, including heavy alcohol abuse, smoking, abdominal trauma, medications, infections, tumors, high triglyceride and calcium levels in the blood and anatomic abnormalities of the pancreas. About 80% of acute pancreatitis cases are due to the presence of gallstones in the gallbladder, which causes an obstruction of the normal flow of pancreatic fluid damaging the acinar cells [3]. The production of inflammatory mediators from damaged acinar cells, as well as from the infiltrated inflammatory cells, leads to local and severe cases of systemic inflammation. Systemic inflammation and multiple organ dysfunction syndrome (MODS), including lung, liver, kidneys and spleen, are the primary causes of morbidity and mortality in acute pancreatitis.

The risk of chronic pancreatitis is greatest among users of alcohol and tobacco. Chronic pancreatitis is characterized by a progressive and irreversible inflammation of the pancreas, which triggers permanent damage of acinar cells due to the development of fibrosis. Consequently, there is an atrophy of acinar cells limiting the secretion of digestive enzymes. Ultimately, it leads to organ failure, intestinal malabsorption, weight loss and endocrine dysfunctions [4]. Besides inducing irreversible pancreatic damage, chronic pancreatitis increases the risk to develop pre-neoplastic lesions and pancreatic cancer [5, 6]. More recently, a new form of pancreatitis has been described, the so-called autoimmune pancreatitis (AIP). AIP is a chronic inflammation caused by the body's immune system attacking the pancreas. This form of pancreatitis is rare and epidemiological data are available only relating to AIP in Japan, where national survey data estimates the prevalence of AIP as 0,82-2,2 per 100,000 individuals [7, 8]. This disease typically involves more men than women older than 50 years of age. Nowadays two subtypes of AIP are recognized, type 1 and type 2. Type 1 AIP is also called IgG4-related disease and affects multiple organs including the pancreas, bile ducts, salivary glands, kidneys and lymph nodes. Serologically, an elevated level of IgG4 is the most sensitive and specific finding in AIP type 1. Histologically, AIP type 1 is characterized by lymphoplasmacytic sclerosing pancreatitis (LPSP). LPSP is associated with: i) abundant levels of infiltrated lymphocytes, predominantly T cells, and plasma cells IgG4-positive. Neutrophils are rare in this type of pancreatitis. ii) Storiform fibrosis lesion, characterized by a deposition of disorientated collagen fibers forming a swirling pattern and, iii) by inflammation of veins that results in permanent closure of the vessel affected [9]. On the other hand,

AIP type 2 seems to be a pancreas-specific disorder. Even though about one-third of people with type 2 AIP have associated inflammatory bowel disease, injury in other organs has not been reported in AIP type 2 [10, 11]. The main feature of this AIP type is the lack of abundant IgG4-positive staining plasma cells. Indeed, patients with type 2 AIP have no serological markers of autoimmunity. However, AIP type 2 is characterized by diagnostic lesions termed granulocytic epithelial lesions (GEL), which are formed by an elevated number of neutrophils that ulcerate and destroy the ductal epithelium. GELs affect medium sized and small ducts, but also may be recognized in the acinar tissue. Contrary to AIP type I, this type of AIP present a moderate level of fibrosis centered on ducts [9]. Symptomatically, both types of AIP are indistinguishable. Many patients complain of abdominal pain, although the frequency and intensity of pain attacks seems to be lower in patients with type 1 AIP than type 2 AIP. Both subtypes of AIP are treated with corticosteroids, which in many patients dramatically improve the condition. However, relapses are common in AIP type 1 and rare in type 2.

2.2.2 Development of inflammation.

The early phases of acute pancreatitis are characterized by two major and independent cellular events: i) a premature intra-acinar activation of trypsinogen, leading to auto digestion and necrosis of the pancreas and ii) activation of NFκB signaling, leading to the development of inflammation [12]. Trypsinogen activation derives from abnormal fusion of lysosomal and zymogen granule compartments. Hence, it triggers the entire zymogen activation cascade with consequent acinar and tissue injury. Trypsinogen activation was believed to be the central pathogenic event of pancreatitis for many decades [13]. However, the inflammatory response triggered by NFκB signaling affects not only the pathogenesis but also the course of the disease [14], as it is the responsible for systemic effects and multi-organ failure. Importantly, both trypsinogen and NFkB activation induce acinar cells to release an important number of inflammatory mediators, such as platelet activating factor (PAF), reactive oxygen species (ROS), transforming growth factor β (TGF-β), nitric oxide (NO), cytokines and chemokines. Besides acinar cells, pancreatic stellate cells (PSCs) and resident macrophages also secrete these pro-inflammatory mediators and play a dynamic role in recruiting leukocytes into the organ. They are essential determinants of local and systemic tissue injury. Amongst the leukocytes recruited, neutrophils aggravate the inflammation by releasing myeloperoxidase, reactive oxygen species (ROS) and proteolytic enzymes into the interstitium. In addition, monocytes and macrophages are also recruited to the pancreas, playing a critical role in the progression of the inflammatory response. They can change their physiology in response to the environment. During the initial stages of pancreatitis, infiltrated macrophages contribute to clearance of damaged cells via phagocytosis, but an impaired clearance of injured acini by macrophages is associated with an altered cytokine production, which stimulates a further immune response and recruitment of new leukocytes [15]. Macrophages can also induce tissue damage mainly via the production of reactive oxygen species. Thus, macrophages can promote the progression of inflammation. Indeed, the degree of macrophage activation is considered to be one of the main factors that determines the severity of acute pancreatitis [14, 16]. Several experimental and clinical reports indicate that the presence of pro-inflammatory stimuli induces classical activation of macrophages (M1 macrophages). During this activation, M1 macrophages are characterized by the secretion of pro-inflammatory cytokines, including TNFα, IL1β and IL6. In addition, they express

enzymes, such as inducible nitric oxide synthase (iNOS) or cyclooxygenase-2 (COX2), involved in the generation of other pro-inflammatory mediators as nitric oxide or arachidonic acid metabolites [17], which promote local and systemic inflammation. Although macrophages are the major leukocyte population found in the inflamed pancreas, other types of lymphocytes, including T lymphocytes and dendritic cells have also been described to play an important role in the development of acute pancreatitis [18, 19]. (Pancreatic inflammation is summarized in Figure 2A).

2.2.3 Pancreatic regeneration.

Organ regeneration is a common biological process involved in the natural turnover of cells. In contrast to physiological regeneration, regeneration under pathophysiological conditions is always preceded by tissue damage. It results in disturbance of normal tissue architecture, loss of parenchymal cells and deposition of cellular debris. Following damage, it is necessary to restore normal organ structure and functionality. This is achieved by the integration, in time and space, of several complex biological processes. These complex mechanisms of regeneration consist of i) cell replication to compensate the loss of cells, ii) pattern formation to renew tissue architecture and ii) inflammation development to remove cellular debris. Rodent and human pancreas can regenerate from mild pancreatitis. Acinar cells have an important role in the regeneration of the exocrine pancreas. Rodent experimental models based on tamoxifen-inducible lineage-tracing, where the fate of acinar cells was compared before and after injury, demonstrated that most of the regenerated acinar cells derive from acinar cells already existing at the time of injury. Hence, these data demonstrated that acinar cell regeneration is driven mainly by proliferation of surviving acinar cells in cerulein-induced experimental pancreatitis or after partial pancreatectomy [20, 21]. However, the question remains whether a more severe injury may provoke an alternative form of regeneration. In this regard, new evidences of a different type of exocrine pancreas regeneration have been published (reference [12, 22]). In this work the authors used a model of regeneration based on severe pancreatic injury caused by diphtheria toxin. This approach induces an extensive ablation of acinar but not ductal cells. In this context, epithelial cells within the ductal compartment contributed to acinar cells regeneration. This regenerative mechanism recapitulated the embryonic pancreatic developmental program where the acinar cells are generated by ductal-to-acinar trans-differentiation rather than by acinar self-renewal. These results suggested that the capacity of epithelial ductal cells to adopt an acinar identity is context-dependent and that the severity of injury determines the regenerative mechanisms and cell types that contribute to this process. These results are very interesting, however further studies are required to understand their relevance in human pancreatic diseases. Another interesting aspect of pancreatic regeneration concerns the formation of acinar-to-ductal metaplasia (ADM), an acinar cell response to inflammatory injury. ADM is a reprogramming event that induces a morphological remodeling of the acinar architecture leading to a structural trans-differentiation of acinar cells into a ductal-like phenotype. This reprogramming consists of an initial downregulation of acinar-specific genes, followed by upregulation of ductal markers and pancreatic progenitor genes that recapitulates aspects of embryonic development [23]. The molecular factors that regulate ADM formation are complex. NFκB signaling pathway is a critical inducer of pancreatic inflammation and ADM [24]. In addition, both infiltrating inflammatory cells and acinar cell DNA damage are also associated with the development of ADM [20] [25]. Furthermore, EGFR signaling

plays a central role during ADM induction and progression in response to inflammatory signals [26] [27]. Usually, ADM formation is a reversible process during pancreatitis. In this context, several signaling pathways, including Notch [28], β-catenin [29], Sonic hedgehog (SHH) [30] and TGFβ [31], promote ADM re-differentiation into acinar cells in the injured organ and its return to normal phenotype. However, several transgenic mouse models showed that persistent ADM is associated with impaired regeneration. Furthermore, in combination with genetic alterations, ADM can evolve into lesions known as pancreatic intraepithelial neoplasia (PanIN), which can further progress into pancreatic ductal adenocarcinoma (PDAC). Thus, the loss of acinar cell re-differentiation drives pancreatic cancer initiation, providing a mechanistic link between pancreatitis and cancer risk. Thus, it is still debated if ADM is required for organ regeneration or it is a side effect of pancreatic inflammation with potential negative implications for regeneration if not timely controlled. (Pancreatic regeneration is summarized in Figure 2B).

2.2.4 Development of fibrosis.

Development of fibrosis is a major histological feature of chronic pancreatitis. It is the result of a progressive necro-inflammatory condition causing irreversible damage to the pancreatic parenchyma. Excessive scarring constitutes the pathogenesis of pancreatic fibrosis. It is due to the production, deposition and contraction of extracellular matrix. In recent times, the identification, isolation and characterization of the cells that mediate the development of pancreatic fibrosis permitted significant progress in understanding this pathology. These cells are called pancreatic stellate cells (PSCs). In the uninjured pancreas, PSCs are quiescent and can be identified by the presence of vitamin A-containing lipid droplets in the cytoplasm (see table 1) [32]. PSCs show a peri-acinar distribution and constitute 4% of all pancreatic cells [33]. In response to pancreatic injury or inflammation, quiescent PSCs become activated and transdifferentiate into myofibroblast-like cells, which express α-smooth muscle actin (α-SMA) (see table 1). Activated PSCs proliferate, migrate, produce extracellular matrix components, such as collagen, and express cytokines and chemokines [33, 34]. PSCs can be activated by a variety of toxic factors associated with pancreatitis, such as alcohol and its metabolites, endotoxin, oxidant stress, hyperglycaemia and hypoxia. In addition, soluble factors, including a variety of cytokines and growth factors, have been described to activate PSCs. In particular, platelet-derived growth factor (PDGF) is the major promoter of PSC migration, while transforming growth factor-β1 (TGF-β1) induces PSC to synthesize and secrete collagen, fibronectin and laminin via a Smad-associated pathway. [35]. Potential sources of these factors are activated macrophages, platelets, pancreatic acinar cells, ductal cells and endothelial cells in the inflamed pancreas. In this context, it is known that acinar cells adjacent to fibrotic areas are strongly positive for TGB-β, suggesting that TGF-β secreted by acinar cells could have a paracrine effect on PSCs, leading to an increased synthesis of collagen by PSCs [36, 37]. Importantly, PSCs by themselves are capable of synthesizing cytokines and chemokines, suggesting the existence of autocrine loops that might contribute to the perpetuation of PSC activation after stimulation by an initial exogenous signal, thus promoting the development of fibrosis [34]. (Development of pancreatic fibrosis is summarized in Figure 2C).

Table 1.

General features of:	QUIESCENT PSCs	ACTIVATED PSCs
Localization	Periacinar location	Fibosis areas
Morphological features		
General appearance	Spindle shaped & smaller	Star shaped & bigger
Nucleus	Basal size	Enlarged
Endoplasmatic reticulum	Limited	Increased
Vitamin A - lipid droplets	Present	Absent
* **Molecular markers**		
Vimentin	++	++
Desmin	+	+
GFAP	+	+
Nestin	+	++
a-SMA expression	-	+++
Properties		
ECM protein secretion	Limited	Increased
Production of cytokines	Limited	Increased
Cell proliferation	Limited	Increased
Cell migration	Limited	Increased
Capacity for phagocytosis	Absent	Present

Adapted from: Omary, Lugea et al. 2007 & Apte, Pirola et al. 2012

Table 1. General characteristics of quiescent and activated pancreatic stellate cells (PSC). - absence; + presence; ++ higlx present; +++ hugely present. *PSCs molecular markers can diverge depend on cell activation state, organ and specie, i.e: 70%-80% of hepatic stellate cells (HSCs) on rat express desmin, whereas human HSCs seem to lack desmin expression.

Figure 2.

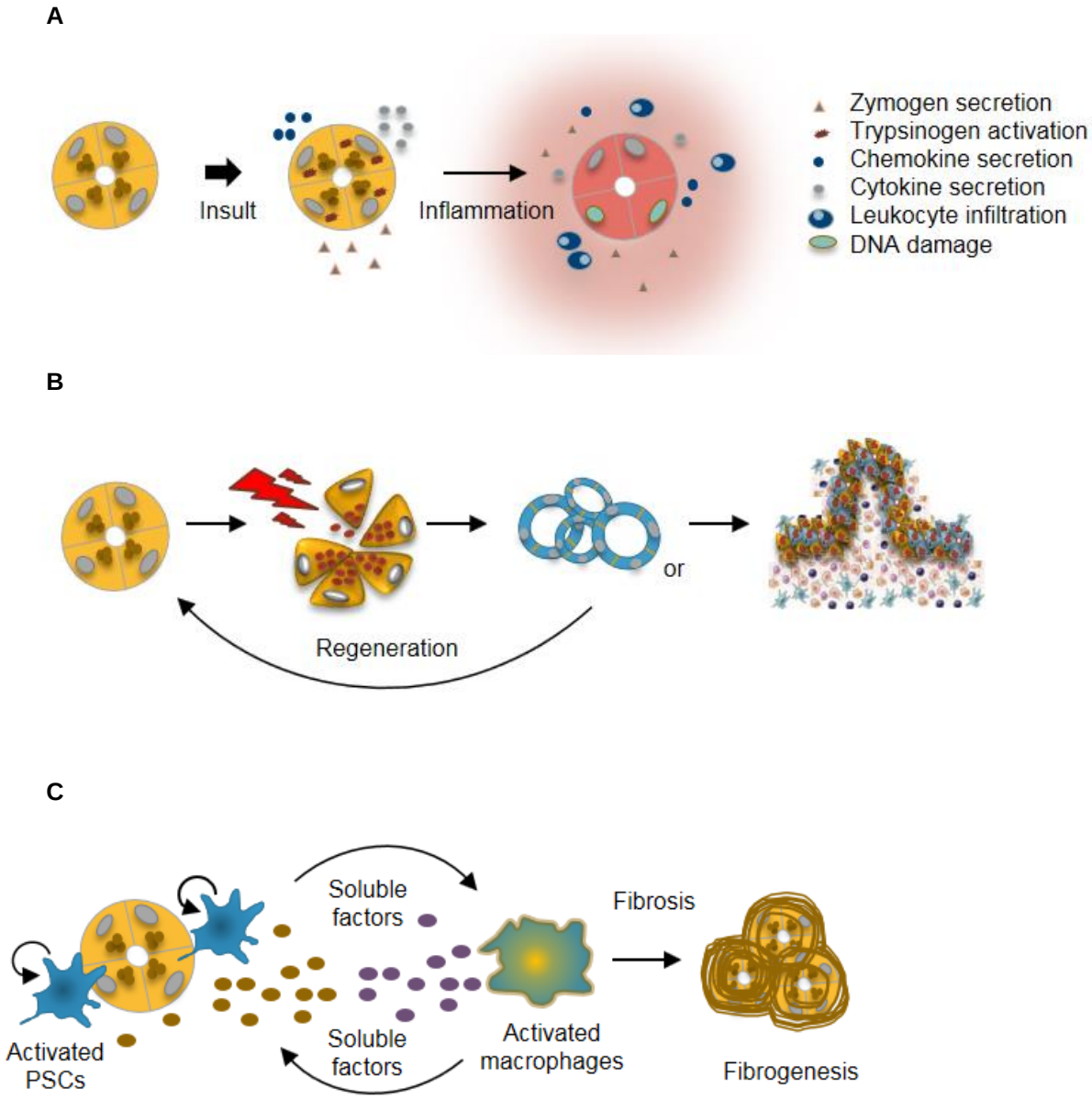

Figure 2A. Schematic representation of the pathophysiology of acute pancreatitis (AP). **Figure 2B.** Schematic representation of ADM formation during AP pancreatitis evolution. ADM lesions can re-differentiation into healthy acinar cells (regeneration) or toward to PaNIN lesions (impaired regeneration). **Figure 2C.** Schematic representation of fibrosis deposition during chronic pancreatitis (CP). Solid lines, direct effects.

2.2.5 Pancreatitis: a therapeutic challenge

There is currently no cure for pancreatitis and the goal of medical management is to provide supportive care. Initial analgesic therapy consists of pain-relieving and anti-inflammatory drugs to decrease pancreatic inflammation. Following the discovery of the central role of PSCs in pancreatic fibrosis, potential treatments have been proposed to target their activation. However, an effective anti-fibrotic therapy has yet to be established. To control malabsorption during chronic pancreatitis, pancreatic enzyme replacement therapy is recommended. Surgery intervention is indicated only in selected cases of acute and chronic pancreatitis to remove necrotic tissue, to correct organ abnormalities and to relieve pain. Thus, pancreatitis is still a clinical challenge and further investigations are urgently required to identify and develop new treatments for this debilitating disease. In this context, recent studies have shown that epigenetic mechanisms are activated in several organs during inflammatory and fibrotic diseases. Specifically, epigenetic modifications based on the activity of histone deacetylases (HDACs) and histone acetyltransferases (HAT) play essential roles in regulating transcription of genes critically involved in inflammation and fibrosis in kidney, liver, heart, and lung [38-43]. Thus, we investigated whether similar epigenetic mechanisms play a role in promoting the pathophysiology of acute and chronic pancreatitis and whether they may provide a novel therapeutic focus.

2.3 Epigenetics

The 20th century was the golden age of genetics. However, nowadays we know that gene expression does not just depend on the DNA sequence. It is also affected by epigenetic factors. The word “epigenetic” literally means “on top of genetic”. Thus, epigenetics is the study of heritable changes in gene expression that do not involve changes to the DNA sequence. Recently, histones have been shown to be dynamic proteins, which undergo multiple types of post-translational modifications to regulate chromatin condensation. This is an important process in the regulation of gene expression as, by altering the electrostatic forces of the chromatin and/or by altering the affinity of interactions with chromatin-binding proteins, histone modifications change DNA accessibility. Acetyl groups (acetylation), methyl groups (methylation), phosphor groups (phosphorylation), ubiquitins (ubiquitylation) and sumo groups (sumoylation) can modify histones. Besides histone modifications, other epigenetic mechanisms initiate and sustain epigenetic changes such as DNA methylation and non-coding RNA (ncRNA) [34].

2.3.1 Histone modification: acetylation vs deacetylation.

Acetylation of histone proteins was first discovered in the early 1960s and it is probably the best understood type of modification [44]. Acetylation occurs on lysine residues and it is a highly dynamic process regulated by the opposing action of two families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs catalyze the transfer of an acetyl group from the acetyl coenzyme A metabolic intermediary to the ε-amino group of certain lysine residues in the basic N-terminal tail region of histones [36]. This HAT action neutralizes the lysine’s positive charge and decreases the interaction of the N-terminal tail regions of histones with the negatively charged phosphate groups of DNA. As a consequence, the structure of chromatin relaxes, thus facilitating access to the transcription machineries and promoting gene transcription. This chromatin relaxation is reversed by HDACs’ activity. HDACs remove acetyl groups from lysines, allowing interactions between

negatively charged DNA and positively charged histone proteins, which results in chromatin compaction and transcriptional silencing of genes. Hence, HDACs are predominantly transcriptional repressors enzymes. With their opposed regulatory activities, HATs and HDACs determine the level of histone acetylation in order to have a regulated gene transcription [37-40] (Figure 3, tables 2 and 3).

Figure 3.

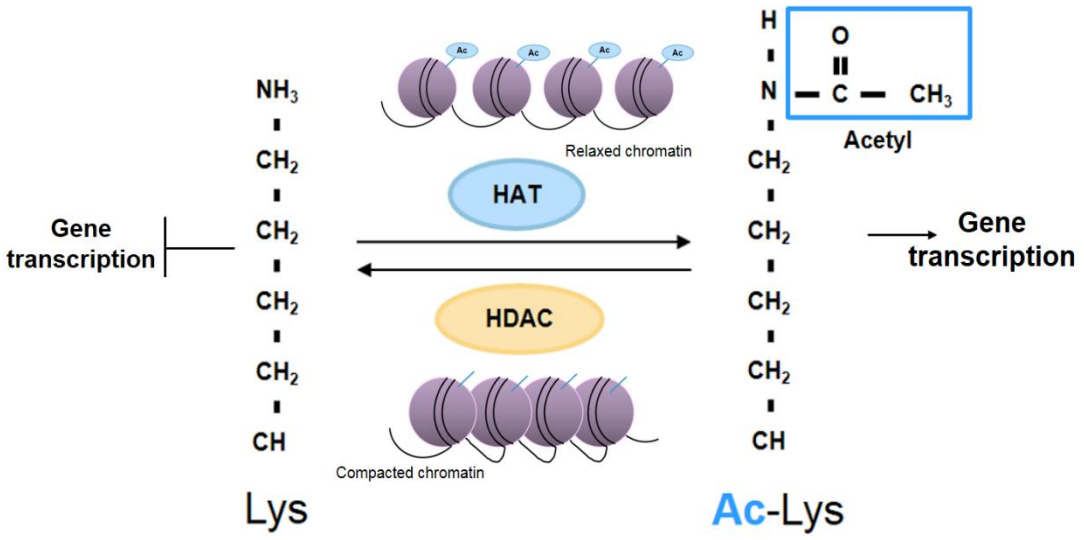

Figure 3. Schematic representation histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymatic activity. Purple balls represents the histones. Blue balls (Ac) represents acetyl groups on the histone tails. Blue dash represents histone tails without Ac group. HAT and HDAC with opposed actions to maintain the steady-state levels of acetylation and control gene regulation. Acetylation (Ac) targets Lys residues in the amino-terminal tails of core histone proteins. Histone tail acetylated relaxes chromatin, and allows transcriptional activation. Histone deacetylate results in closed-chromatin configuration thereby blocking the access to transcription factors and other transcription co-activators inducing transcriptional repression.

Since the discovery of HDACs the epigenetic field has progressed rapidly. Several epigenetic studies have demonstrated that fundamental developmental signaling transduction pathways, that are quiescent in the adult organ, reemerge during organ injury as well as in tumorigenesis [23, 45]. During these processes, alterations of HDACs' expression has been observed, concomitant with chromatin modifications and chromosome segregation defects, which could induce disease initiation and progression. The role of HDACs in the pathophysiology of several diseases opens up the possibility that epigenetic drugs may have a powerful impact on their treatment. Indeed, this possibility has been confirmed for cancer therapy. Food and Drug Administration has approved several HDAC inhibitors (HDACi) against specific types of cancer. These epigenetics drugs have been authorized based on their effectiveness in inhibiting tumor growth, their capacity to induce specific changes in gene expression

and their influence on a variety of processes including differentiation, growth arrest, cytotoxicity and induction of apoptosis. Although HDACi were initially used as anti-cancer agents, they are now becoming a promising therapeutic approach for non-malignant diseases, including inflammatory diseases [39, 46]. Furthermore, recent data confirm that changes in the acetylation status of histones and abnormal expression and activation of antagonistic HATs and HDACs regulate inflammatory gene expression [47]. Indeed, *in vitro* and *in vivo* studies using HDACi have already shown positive therapeutic effects in a variety of animal models of inflammatory diseases, such as arthritis, inflammatory bowel diseases, ischemia-reperfusion injury, asthma, diabetes, acute kidney injury, spinal cord inflammation, demyelination, neuronal and axonal loss in experimental autoimmune encephalomyelitis [46, 48-50]. In addition to this anti-inflammatory effect, a growing body of evidence shows that epigenetic modulation through HDACi can have anti-fibrotic effects. Specifically, HDACi counteract the development of fibrosis in lung, liver, kidney and skin [39, 51-54].

Table 2.

HAT class	Family	Structural features	Location	Action
Type A	GNAT	Bromodomain	N	Acetylate histone in chromatin
	p300/CBP	Bromodomain	N	Acetylate histone in chromatin
	$TAF_{II}250$ (TAF1)	Bromodomain	N	Acetylate histone in chromatin
Type B	MYST	Chromodomain	C	Acetylate free histones

Table 2. Histone Acetyltransferas (HAT) classification. There are two major classes of HATs described. HATs type-A and HATs type-B. The type-B are predominantly located in the cytoplasm acetylating free histones. The type-A HATs are a more diverse family than the type-B. The type-A HATs are classified into three separates groups depending on amino-acid sequence homology and conformational structure. Each of these enzymes modifies multiples sites within the histone N-terminal tails.

Table 3.

HDAC Class	Type	Subcellular localitzation	Location in body	Co-factor	Catalytic domain
Class I	HDAC 1	N	Ubiquitous	Zn^{2+}	
	HDAC 2	N	Ubiquitous	Zn^{2+}	
	HDAC 3	N> C	Ubiquitous	Zn^{2+}	
	HDAC 8	N & C	Ubiquitous	Zn^{2+}	
CLASS II a	HDAC 4	N & C	Tissue specific	Zn^{2+}	
	HDAC5	N & C	Tissue specific	Zn^{2+}	
	HDAC 7	N, C & M	Tissue specific	Zn^{2+}	
	HDAC 9	N& C	Tissue specific	Zn^{2+}	
CLASS II b	HDAC 6	C > N	Tissue specific	Zn^{2+}	
	HDAC 10	C > N	Tissue specific	Zn^{2+}	
CLASS III Sirtuins	SIRT (1-7)	N, C & M	Tissue specific	NAD^{+}	
Class IV	HDAC 11	N	Tissue specific	Zn^{2+}	

Table 3. Histone deacetylase (HDAC) classification. Isoforms of HDACs are divided into four classes based on the sequence similarity of their catalytic domain to yeast homologs. The distribution of the conserved catalytic domain and dominant region of subcellular localization are indicated. N; means Nucleos. C; means cytoplasm. M; means Mitochondria.

Aims of the project

- To investigate whether HDAC activity is up-regulated during different phases of pancreatitis development.
- To assess the therapeutic value of class I HDAC inhibition during the development of pancreatitis.

3. Aim of the project.

The functions of HDACs in the pancreas are still largely unexplored. A very interesting study revealed that HDAC activity is high in the embryonic pancreas and it plays a critical role in the development of the organ by orchestrating the gene expression that dictates the lineage of different cell types [55]. So far, the role of HDAC activity in the adult organ during diseased conditions, in particular pancreatitis, has not been thoroughly investigated. The few studies performed using different HDACi and different experimental models of pancreatitis presented conflicting results. Specifically, two recent studies showed that *in vivo* treatment with the pan-HDAC inhibitors sodium butyrate or trichostatin A (TSA), targeting both class I and class II subfamilies, alleviated pancreatic damage, inflammation and fibrosis following L-arginine [54] or taurocholic acid-induced pancreatitis [56] in rodents. However, another recent publication showed that treatment with the antiepileptic compound valproic acid (VPA), also known for its function as HDACi, increased pancreatic injury and it reduced the normal regenerative capacity of pancreatic acinar cells following cerulein-induced pancreatitis. [53]. Thus, further studies are needed to determine the function of HDACs during the development and resolution of pancreatitis. To achieve this goal, we aim to:

- **Investigate whether HDAC activity is up-regulated during different phases of pancreatitis development.**

  For this purpose, we evaluated the temporal regulation of HDACs in the different phases of the disease. First, we focussed on the early aspects that characterize the first week of pancreatitis (acute phase). Then, we evaluated the progression of the disease over six weeks of time (chronic phase). For these analyses, we induced pancreatitis by repetitive cerulein injections. Cerulein is a stable analogue of the hormone cholecystokinin (CCK), which, when administered at supra-maximal concentrations, induces aberrant pancreatic enzyme secretion, acinar cell damage and inflammatory phenotype typical of human pancreatitis. Finally, we investigated HDAC expression in the development of autoimmune pancreatitis. In this case, we used transgenic mice overexpressing lymphotoxin (LT) α and β in pancreatic acinar cells that spontaneously develop the disease at 12 months of age [57]. Using all these mouse models, we were able to characterize HDACs expression during the diverse phases and types of pancreatitis.

- **Assess the therapeutic value of class I HDAC inhibition during the development of pancreatitis.**

  For this objective, we used the potent selective inhibitor of class I HDAC MS-275, also called Etinostat, a synthetic derivative of 2-aminophenyl benzamide. Nowadays there is growing interest for this specific epigenetic drug. Indeed, MS-275 is being tested in clinical phase III trials for receptor-positive advanced breast cancer and in phase II trials for lung cancer, breast cancer, and Hodgkin lymphoma [58]. In our case, the inhibitor was administered in both preventive and therapeutic regimens and its effects were evaluated during the acute

and chronic phases of the disease. Specifically, we focussed on the main features that characterize the progression of pancreatitis, namely initial damage of acinar cells, development of inflammation, acinar cell proliferation, formation of acinar-to-ductal metaplasia and organ fibrosis.

To achieve the aims, i.e. the expression and activity of HDACs, the effects of class I HDAC inhibition with the selective inhibitor MS-275 during the different phases of pancreatitis and the identification of the underlying molecular mechanisms, we used *in-vivo* and *in-vitro* approaches, employing biochemical, qRT-PCR and imaging techniques.

Class I HDAC inhibition improves pancreatitis outcome by limiting leukocyte recruitment and acinar-to-ductal metaplasia.

Marta Bombardo, Enrica Saponara, Ermanno Malagola, Rong Chen, Gitta M. Seleznik, Cecile Haumaitre, Evans Quilichini, Anja Zabel, Theresia Reding, Rolf Graf and Sabrina Sonda.

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Manuscript A

Class I HDAC inhibition improves pancreatitis outcome by limiting leukocyte recruitment and acinar-to-ductal metaplasia

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Short title: MS-275 ameliorates the outcome of pancreatitis

Conflicts of interest: The authors disclose no conflicts.

ABSTRACT

Background and Purpose
Pancreatitis is a common inflammation of the pancreas with rising incidence in many countries. Despite improvements in diagnostic techniques, the disease is associated with high risk of severe morbidity and mortality and there is an urgent need for new therapeutic interventions. In this study we evaluated whether histone deacetylases (HDACs), key epigenetic regulators of gene transcription, are involved in the development of the disease.

Experimental Approach
We analyzed HDAC regulation during cerulein-induced acute, chronic and autoimmune pancreatitis using different transgenic mouse models. The functional relevance of class I HDACs was tested with the selective inhibitor MS-275 *in vivo* upon pancreatitis induction and *in vitro* in activated macrophages and primary acinar cell explants.

Key Results
HDAC expression and activity were up-regulated in a time-dependent manner following induction of pancreatitis, with the highest abundance observed for class I HDACs. Class I HDAC inhibition did not prevent the initial acinar cell damage. However, it effectively reduced the infiltration of inflammatory cells, including macrophages and T cells, in both acute and chronic phases of the disease, and directly perturbed macrophage activation. In addition, MS-275 treatment reduced DNA damage in acinar cells and limited acinar de-differentiation into acinar-to-ductal metaplasia in a cell-autonomous manner by impeding the EGFR signaling axis.

Conclusions and Implications These results demonstrate that class I HDACs are critically involved in the development of acute and chronic forms of pancreatitis and suggest that blockade of class I HDAC isoforms is a promising target to improve the outcome of the disease.

Abbreviations
HDACs (histone deacetylases), ADM (acinar-to-ductal metaplasia), HDACi (HDAC inhibitor), TSA (Trichostatin A), GPT (glutamic-pyruvic transaminase), APCs (antigen presenting cells), H&E (hematoxylin and eosin), TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling, pH3 *(Phospho-Histone H3*), LT (lymphotoxin), CDKi (Cyclin-Dependent Kinase inhibitors) (CDKi), LPS (*Lipopolysaccharide), i.p. (*intraperitoneal).

INTRODUCTION

Abnormal activity of histone deacetylases (HDACs), a family of enzymes involved in the epigenetic control of gene transcription, has been implicated in the etiology and development of several malignancies. However, recent evidence indicates that HDACs are also involved in the development of inflammatory diseases, highlighting the potential of targeting the activity of HDACs as a therapeutic approach to improve the outcome of rheumatoid arthritis [1, 2], neuritis [3, 4], cholitis [5] and autoimmunity [6].

HDACs control the acetylation status of histone and non-histone proteins, thus regulating the expression of target genes (reviewed in [7]). The complexity of acetylation-based epigenetic regulation is reflected by the size of the mammalian HDAC family, composed of eleven zinc-dependent enzymes divided into three subfamilies (classes I, II, and IV) and seven NAD-dependent enzymes in a fourth (sirtuin) subfamily (class III), with non-redundant functions.

The function of HDAC subfamilies in the pathophysiology of pancreatitis, a highly debilitating inflammatory disease with potentially lethal outcome, has not been thoroughly investigated. During pancreatitis the organ is infiltrated by different leukocyte populations, with the most abundant being neutrophils, macrophages, T cells and dendritic cells. Differentiation and activation of these cell types are regulated by HDAC activity [8-10]. In addition, acinar cell proliferation and consequent organ regeneration is observed following inflammatory damage. This regenerative process is characterized by reactivation of a developmental-like program, the regulation of which is still elusive. Based on these observations, we hypothesize that HDAC activity may be a key factor in modulating not only leukocyte infiltration in the injured tissue but also gene expression changes in acinar cells affecting pancreatic regeneration. In support of this hypothesis, two recent studies showed that *in vivo* treatment with the pan-HDAC inhibitors sodium butyrate or trichostatin A (TCA), targeting both class I and class II HDAC subfamilies, alleviated pancreatic damage, inflammation and fibrosis following L-arginine [11] or taurocholic acid-induced pancreatitis [12] in rodents. However, another study reported a detrimental effect upon treatment with valproic acid during cerulein-induced pancreatitis, with consequent delay in tissue recovery and reduced acinar cell proliferation [13]. These conflicting results highlight the need to further investigate the precise role of HDAC isoforms in this disease.

In this study we dissected the temporal regulation of HDACs in different murine models of acute, chronic and autoimmune pancreatitis. Furthermore, we evaluated the therapeutic potential of class I HDAC inhibition to modulate pancreatic inflammation and tissue damage.

METHODS

Animal experiments

All animal experiments were performed in accordance with Swiss Federal animal regulations and approved by the cantonal veterinary office of Zurich. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals. Mice used in this study were adult 8–10 week old wild-type C57BL/6 mice (Envigo Laboratories, Horst, The Netherlands), tryptophan hydoxylase-1 knocked-out mice ($Tph1^{-/-}$)[14], mice with selective TGF-β receptor II ablation in pancreatic epithelial cells ($Ptf1a^{cre/+};Tgf\text{-}\beta\ RII^{fl/fl}$)[15], lymphotoxin αβ-overexpressing mice [16] and cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ knocked-out mice ($p21^{-/-}$) Jackson Laboratories, USA. Acute pancreatitis was induced via six intraperitoneal (i.p.) injections of 50 μg/kg cerulein, administered hourly on two consecutive days. Control animals received 0.9% NaCl injections. MS-275 (Selleckchem, Houston, USA) was injected daily i.p. at 20mg/kg or 40mg/kg, starting

one day before the first cerulein injection. Control animals received vehicle DMSO injections. Chronic pancreatitis was induced via six i.p. injections of 50 μg/kg cerulein, administered hourly three times a week on alternate days (Monday, Wednesday, Friday) for up to six weeks. MS-275 was also administered three times a week on alternate days for two weeks starting concomitantly (preventive regimen) or one week after the beginning of cerulein injections (therapeutic regimen). Except for the mice harvested up to 8 hours after first cerulein-injection, animals did not receive cerulein or MS-275 on the harvest day. For autoimmune pancreatitis we used mice overexpressing lymphotoxin αβ that develop the disease spontaneously at 12 month of age [16].
Groups of 5 animals were tested for each experiment. Animals were assigned randomly to different experimental groups for all *in vivo* studies. Data collection and evaluation of all *in vivo* and *in vitro* experiments were performed blindly of the group identity.

Mammalian cell cultures
Acinar explants were obtained from 4-6 week old wild-type C57BL/6 mice. Cultures of primary acinar explants were isolated according to [17] and embedded in collagen. De-differentiation into ADM was induced by addition of 50 ng/ml recombinant hTGFα (R&D Systems) for five days. Cultures were maintained in Waymouths MB 752/1 medium supplemented with 50 U/mL penicillin, and 50 μg/mL streptomycin, 0.1% fetal bovine serum (FBS), 0.1 mg/ml soybean trypsin inhibitor, and 1 μg/ml dexamethasone, with daily medium replacement. For RNA quantification following de-differentiation, explants grown in 12 well plates were isolated from the collagen matrix using 10 mg/mL collagenase (Sigma) for 15min at 37°C. Samples from four replicas of each group were pooled for RNA extraction as described below.
RAW264.7 macrophage cells were maintained in Dulbecco's Modified Eagle medium (DMEM)+GlutaMAX supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were pre-incubated with 1 μM MS-275 for 30 minutes and stimulated with 10 ng/mL LPS for six hours in the presence or absence of 1 μM MS-275.
Cell number, cell viability and cell diameter were determined using an automated cell counter (NucleoCounter® NC-200™, Chemometec, Allerod, Denmark).
2*10E6 primary macrophages were isolated from the peritoneum of four mice, pooled, seeded in six well plates for two hours and stimulated as described above for RAW cells. At the end of the treatment, cells were lysed in the plate for RNA extraction.

Immunohistochemistry
Pancreas specimens were embedded in paraffin for histological analyses, as described[18]. Primary antibodies used in this study were: rabbit anti-Ki67 (#ab16667, Abcam, Cambridge, UK); rabbit anti-phospho-histone 3 (#2066052, Millipore, MA, USA); rabbit anti-amylase (#A8273-1VL, Sigma-Aldrich, Buchs, Switzerland); rabbit anti-PU.1 (#2266, Cell Signaling Technologies, Danvers, MA); rabbit anti-acetyl-lysine (AcK) (#ab80178, Abcam); rabbit anti-phospho-histone H2A.X (Ser139) (#9718, Cell Signaling Technologies, Danvers, MA); rabbit anti-coronin-1 (gift from Jean Pieters), Rabbit Anti-phospho-EGFR (Tyr1086) (Millipore); rabbit anti CD3 (#A 0452, Dako). Secondary antibodies used in this study were: Alexa Fluor 594 (A-11012, Thermo Fisher, Illkirch, France) and biotinylated goat anti-rabbit IgG (H + L), included in the Vectastain® ABC Kit (PK-4001). Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI).
Detection of DNA fragmentation in apoptotic cells was performed with a TUNEL assay using an ApopTag peroxidase Kit (MP Biomedicals, Illkirch, France).
Microscopy analyses were performed on a wide-field Nikon Eclipse Ti (Amsterdam, The Netherlands). Quantification of labelled cells was performed in at least 10 randomly

selected high-power fields (×200) per slide using the NIS Elements BR Analysis and Cell^P analysis softwares.

Western blotting

Immunoblotting was performed by homogenizing tissue samples in RIPA buffer containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using a Bradford protein assay (BioRad, Hercules, CA, USA). 40 µg protein aliquots were separated by SDS-PAGE electrophoresis and blotted using a V3 Western Workflow system (BioRad, Hercules, CA, USA) according to manufacturer protocols. PVDF membranes were incubated with primary antibodies (rabbit anti-EGFR (# ab52894, Abcam); rabbit anti-α-Tubulin (#2125, Cell signaling) overnight at 4^{o}C. All results were measured by densitometry and expressed by relative expression to tubulin as a reference protein.

Nuclear protein extract and HDAC activity

Nuclear proteins were extracted from 20 mg of pancreatic tissue with the EpiQuik™ Nuclear Extraction Kit (Epigentek Group Inc, Mountain View, CA) and HDAC activity was measured in the nuclear extracts with the fluorimetric EpiQuik HDAC Activity/Inhibition Assay Kit (Epigentek Group Inc. Mountain View, CA), following the manufacturer's instructions.

Transcript analysis

Total RNA was extracted from pancreatic tissue, acinar explants and cell lines, as described previously,[19] and reverse-transcribed with qScript™ cDNA SuperMix (Quanta Biosciences). Gene expression was measured by real-time PCR on a 7500 Fast Real-Time PCR System (Applied Biosystems) using Taqman probes (Applied Biosystems). Transcript levels were normalized using 18S or HRPT RNA as a reference and expressed as ΔCt or ΔΔCt relative to the value of untreated control animals.

Biochemical analyses of enzyme activity

Levels of amylase and glutamate pyruvate transaminase (GPT) were measured in blood collected via heart puncture. Enzymes were measured using the Fuji Dri-Chem 4000i analyzer (FUJIFILM Corporation, Tokyo, Japan).

Trypsinogen activation was measured in frozen pancreatic tissue as described previously [15].

Statistics

Groups of 5 animals were tested for each experiment. The data are expressed as means ± SEM. The statistical significance of differences in the means of experimental groups was determined using an unpaired, two-tailed Student's *t* test or one-way analysis of variance followed by Dunnett post test (GraphPad Prism 4.0c; GraphPad Software, Inc.) and a probability value <0.05 was considered statistically significant.

RESULTS

HDAC activity is up-regulated in the pancreas during acute, chronic and autoimmune pancreatitis

To test whether HDACs are regulated during acute pancreatitis, we induced the disease with serial cerulein injections according to the scheme depicted in Fig. 1A. Class I HDAC1-3 transcripts were the most abundant in the untreated adult pancreas (Fig. 1B), as previously reported [20], and the most up-regulated isoforms following pancreatitis. Increased HDAC expression was transient and returned to baseline levels over time for the majority of HDACs. HDAC activity also increased in the nuclei of pancreatic cells (Fig.1C), while nuclear acetylation decreased (Fig. 1D). Increased HDAC expression did not reflect the increased presence of leukocyte in the pancreas, as HDAC up-regulation i) preceded the peak of immune cell infiltration (Fig. S1A-C), ii) was detected also in isolated acinar cells 24h after induction of pancreatitis (Fig. S2A), and iii) was comparable in control and tryptophan hydoxylase-1 knock-out mice (Fig. S2B), or

TGF-β receptor II knock-out mice (Fig. S2C), which we previously characterized with reduced [21] or increased [22] inflammation, respectively. Finally, HDAC up-regulation was restricted to pancreatic tissue, and unchanged in other organs, including spleen, lungs, liver and brain 24h after induction of pancreatitis (Fig. 1E). We then characterized HDAC expression during the course of chronic pancreatitis. Cerulein administration for up to six weeks, according to the scheme in Fig. 1F, resulted in progressive inflammation of the organ measured by up-regulation of inflammatory markers (Fig. S3A) and tissue damage (Fig. S3B). HDAC expression increased in a time dependent manner, reaching a plateau after four weeks of pancreatitis, with transcripts of class I HDACs being the most abundant (Fig. 1G). Increased HDAC expression was accompanied by increased enzyme activity (Fig. S3C). Finally, we investigated whether HDAC expression is regulated during the course of autoimmune pancreatitis (AIP). To this aim we used transgenic mice overexpressing lymphotoxin (LT) α and β in pancreatic acinar cells that spontaneously develop AIP at 12 months of age [16]. Besides developing autoantibodies, these animals are characterized by increased inflammatory infiltration, expression of pro-inflammatory mediators (Fig. S3D) and formation of tertiary lymphoid organs (TLOs) in the pancreas (Fig. S3E). Similar to what we observed during cerulein-induced pancreatitis, expression of HDAC, particularly of class I, was also up-regulated during AIP (Fig. 1H). Collectively, these data indicate that acute, chronic and autoimmune forms of pancreatitis trigger an increase of HDAC expression and activity in the pancreas.

Inhibition of class I HDAC with MS-275 reduces pancreatic inflammation following induction of acute and chronic pancreatitis.

As members of class I HDACs showed the highest expression during the induction of pancreatitis, we first tested their functional relevance in the acute phase of the disease by administering the selective class I inhibitor MS-275 (Entinostat) according to the regimen in Fig. 2A. MS-275 treatment alone neither altered pancreatic tissue morphology (Fig. S4A) nor induced pancreatic (Fig. S4B) or liver damage (Fig. S4C). Following induction of pancreatitis, MS-275 abrogated pancreatic HDAC activity (Fig. 2B), without reducing the expression of HDAC transcripts (Fig. S5). Parameters of early cell damage, including levels of serum amylase (Fig. 2C), serum glutamic-pyruvic transaminase (GPT) (Fig. 2D), activation of acinar trypsinogen (Fig. 2E) and apoptosis of acinar cells (Fig. 2F) were not reduced in the presence of MS-275. Similarly, expression of CXCL2 and MCP-1, potent chemotactic proteins for neutrophils and macrophages/lymphocytes, were not reduced by MS-275 treatment (Fig. 2G). These data indicate that class I HDAC inhibition does not limit the initial cell damage triggered by cerulein-induced pancreatitis. Despite this phenotype, MS-275 treatment decreased the infiltration of leukocytes in the pancreas (Fig. 2H, S6A), without reducing the expression of several pro-inflammatory molecules (Fig. S6B). Specifically, MS-275 decreased pancreatic levels of activated F4/80-positive macrophages (Fig. 2I), without affecting their polarization (Fig. 2J), and of CD3-positive T cells (Fig. 2K, L), indicating that the inhibitor targets the infiltration of different leukocyte populations.

The anti-inflammatory effect of MS-275 was also tested in the chronic setting of the disease after four weeks of cerulein injections (Fig. 1F), as this time point is characterized by sustained inflammation and extended tissue damage (Fig. S3). We compared a preventive regimen, where MS-275 was administered for two weeks starting concomitantly with cerulein injections, and a therapeutic regimen more relevant for a clinical setting, where MS-275 treatment started a week after induction of pancreatitis (Fig. 3A). Both preventive and therapeutic regimens improved pancreatic morphology (Fig. 3B), significantly reduced pancreatic infiltration of leukocytes (Fig. 3C),

including macrophages (Fig. 3D, E) and CD3-positive T cells (Fig. 3E, F) and decreased the expression of pro-inflammatory cytokines and adhesion molecules (Fig. 3E).

Inhibition of class I HDAC with MS-275 alters the activation of macrophages.

As MS-275 treatment reduced macrophage infiltration during acute and chronic phases of pancreatitis, we then investigated whether MS-275 directly affects the activation of this cell type. Activation of RAW264.7 macrophages with lipopolysaccharide (LPS) increased the expression of HDAC1 (Fig. 4A) and HDAC activity (Fig. 4B). Pre-incubation with 1 μM MS-275 reduced the LPS-induced HDAC activity (Fig. 4B) without affecting cell viability (Fig. 4C). Moreover, MS-275 treatment altered RAW264.7 macrophage activation, as seen by limited LPS-induced size increase (Fig. 4D), and deregulated the expression of selected inflammatory molecules, including decreased IL6 and increased IL1β levels (Fig. 4E). A similar deregulation of transcript expression was also observed in primary peritoneal macrophages stimulated with LPS in the presence of MS-275 (Fig. 4F).

MS-275 treatment results in reduced acinar replication following acute and chronic pancreatitis

Following an inflammatory insult, acinar cells initiate a proliferation program to regenerate the damaged tissue. MS-275 treatment decreased the number of replicating acinar (Fig 5A, B) and interstitial cells (Fig 5B, C). Expression of late cyclins A and B was also reduced (Fig. 5D), while early cyclin D and E expression was unchanged (Fig. S7A). Reduced replication was not paralleled by a general up-regulation of cyclin-dependent kinase inhibitors (CDKi). MS-275 treated pancreata showed a trend of increased expression only for the CDKi $p21^{cip1/WAF}$ (Fig. 5E), while the expression of other CDKis did not change or was reduced upon MS-275 treatment (Fig. S7B). To further investigate the role of p21 in the observed MS-275 phenotype, we tested the effect of the inhibitor in p21 deficient ($p21^{-/-}$) mice upon induction of acute pancreatitis. Similar to what was observed in wild type mice, MS-275 reduced macrophage infiltration (Fig. 5F), acinar cell replication (Fig. 5G) and late cyclin expression, without up-regulating CDKi levels (Fig. 5H). These results suggest that the reduced cell replication upon MS-275 treatment is not a consequence of increased CDKi expression. Finally, MS-275 treatment during chronic pancreatitis also resulted in reduced replication of acinar and interstitial cells (Fig. 5I) and decreased expression preferentially of late cyclins (Fig. 5J).

Inhibition of class I HDAC ameliorates pancreatic tissue damage following acute and chronic pancreatitis

The reduced number of replicating acinar cells upon MS-275 treatment may be a consequence of decreased acinar injury linked to the reduced inflammatory infiltration. In support of this hypothesis, DNA damage in acinar cells, known to be triggered by inflammation (reviewed in [23]), was lower in both MS-275-treated C57BL/6 and $p21^{-/-}$ mice, as shown by quantification of γH2AX (Fig. 6A), an early hallmark of active DNA damage response. Acinar DNA damage [24] and macrophage infiltration [25] are also associated with the development of acinar-to-ductal metaplasia (ADM), a transient de-differentiation of acinar cells occurring during the regenerative phase of pancreatitis. MS-275 reduced ADM formation 72h after induction of pancreatitis (Fig. 6B). During ADM formation, acinar cells lose their mature phenotype, including amylase content, and express progenitor-like genes, such as Sox9. In untreated condition, Sox9 is detectable in the nuclei of centro-acinar and ductal cells; following an inflammatory insult, Sox9 is also transiently expressed by acinar cells, ductules and ADM (representative pictures of Sox9 staining are shown in Fig. 6C). Staining for Sox9 revealed that MS-275 treatment reduced not only the amount of

ADM lesions but also the expression of Sox9 in acinar cells (Fig. 6D).
We further tested the effect of class I HDAC inhibition on tissue damage after four weeks of cerulein treatment. In the setting of chronic pancreatitis, parenchima degradation is more evident than in the acute phase of the disease, where ADM are present as focal lesions, and it is characterized by widespread areas with clusters of interstitial cells and ADM interspersed amongst intact acinar cells containing amylase. Quantification of damaged tissue showed that both preventive and therapeutic MS-275 regimens effectively reduced the level of tissue damage (Fig. 6E) and the expression of progenitor-like genes (Fig. 6F). Moreover, we investigated whether factors promoting acinar de-differentiation into ADM were down-regulated by MS-275 treatment. EGF-mediated signaling pathway is a potent inducer of ADM and malignant transformation [26]. We found that MS-275 reduced the expression of EGF receptor (EGFR) in the pancreas at transcript (Fig. 6G) and protein levels (Fig. 6H) and the amounts of its phosphorylated active form (Fig. 6I).

Inhibition of class I HDAC reduces the formation of ADM in a cell autonomous manner

Reduced EGFR expression upon MS-275 treatment could derive from reduced production of EGFR ligands and receptor expression and activation or from a direct inhibition of EGFR expression in acinar cells by MS-275. To test the latter hypothesis, we triggered acinar cell explants de-differentiation into ADM by EGFR stimulation with TGFα. EGFR stimulation for five days was accompanied by up-regulation of HDAC isoforms, with class I displaying the highest expression increase (Fig. 7A). MS-275 treatment reduced the formation of ADM in a dose-dependent manner (Fig. 7B) and prevented the up-regulation of EGFR and ADM markers, such as cytokeratin 19 and Sox9 (Fig. 7C). Abrogation of ADM formation was not the result of acinar cell death, as acinar clusters that did not de-differentiate were metabolically active (Fig. 7D). These data indicate that class I HDAC inhibition prevents acinar de-differentiation into ADM in a cell autonomous manner and that the effect is likely mediated by a down-regulation of the EGFR expression in acinar cells.

DISCUSSION

In this study we demonstrated that HDAC activity is up-regulated in the pancreas during acute, chronic and autoimmune pancreatitis in different mouse strains. Moreover, pharmacological inhibition of class I HDAC ameliorated the outcome of both acute and chronic forms of pancreatitis by reducing leukocyte infiltration and limiting tissue damage and acinar cell de-differentiation into ADM. These results directly link the activity of class I HDACs with the pathophysiology of this severe disease.

MS-275 and pancreatic inflammation

The reduced inflammation observed following MS-275 treatment may result from different mechanisms. We found that, differently to what was observed upon pan-HDAC inhibitor treatment [12], MS-275 neither prevented the initial acinar damage nor the expression of chemokines necessary to recruit leukocytes. However, the inhibitor could directly affect leukocyte populations. Indeed, MS-275 altered the activation of macrophages and resulted in defective recruitment of these cells into the pancreas. Our results complement previous *in vitro* studies showing that MS-275 modifies the expression of inflammatory molecules and suppresses the migratory properties of antigen presenting cells (APCs), particularly macrophages and dendritic cells [10, 27]. As activated APCs regulate the activation of distinct inflammatory cell types, including T helper cells (Th1, Th2), T regulatory cells, neutrophils, monocytes, and B lymphocytes, it is clear that impairment of APC activation via HDAC inhibitors harbour the potential to inhibit immune processes at multiple levels.

Indeed, we also observed a reduced infiltration of CD3-positive T cells upon MS-275 treatment.
Interestingly, the inhibitor down-regulated IL6, while up-regulated IL1β in both RAW264.7 and primary macrophages. The biological significance of the different cytokine expression remains to be further elucidated, as they are able to exert both pro-inflammatory and anti-inflammatory actions. Nevertheless, it is worth mentioning that IL1β up-regulation was recently shown to induce apoptosis in bacterial-infected macrophages by inducing TNF [28]. Albeit we did not detect overt cell death of macrophages upon MS-275 treatment in the short term (hours) of our experimental setting, it is possible that long term (days) MS-275 incubation during pancreatitis may promote macrophage apoptosis and consequent reduced infiltration of these cells in the pancreas.

MS-275 and ADM formation

Our results revealed not only that acinar de-differentiation into ADM was accompanied by increased HDAC expression but also that class I HDAC inhibition with MS-275 was sufficient to prevent ADM formation both *in vitro* and *in vivo*. In addition, our analyses highlighted the EGF receptor (EGFR) signaling pathways as the underlined molecular mechanism targeted by class I HDAC inhibition. Specifically, we found that MS-275 treatment reduced the expression of EGFR in both *in vivo* and *in vitro* models, resulting in decreased acinar de-differentiation. These findings support the concept that increased class I HDAC activity in acinar cells is required to induce their de-differentiation into ADM by cell-autonomous mechanisms. The described HDAC-mediated regulation of acinar de-differentiation in the adult pancreas recapitulates what was previously identified during pancreatic development. In this context, global decrease in HDAC expression and activity, as well as increase in histone acetylation were observed in the organ during the transition from embryonic to adult stages. Not only cell differentiation into the various pancreatic cell types was concomitant with HDAC down-regulation, but also cell differentiation was modified by HDAC inhibition, therefore identifying the concerted and time dependent HDAC activity as a crucial regulatory factor for lineage commitment of the different pancreatic cell types [20, 29].
Taking into consideration that i) HDAC activity supports acinar de-differentiation during development and in the adult state following inflammatory insult, ii) prolonged de-differentiation of acinar cells into ADMs is detrimental for pancreatic recovery following pancreatitis and iii) ADMs are considered pre-malignant lesions associated with the risk to develop pancreatic cancer, it is tempting to speculate that HDAC inhibitors harbour great potential as therapeutic agents in pancreatic inflammation. However, care has to be applied in the choice of the HDAC inhibitor, as treatment with valproic acid, an anti-epileptic drug that also shows anti-HDAC activity, has been associated with development of pancreatitis in humans [30] and with disease exacerbation in mice, manifested by increased inflammation, decreased proliferation and persistent acinar de-differentiation with non-resolving ADMs [13].

MS-275 and acinar cell proliferation

Our results also revealed that MS-275 treatment during pancreatitis was associated with a reduced number of proliferating acinar cells. This phenotype is likely to result from reduced tissue and DNA damage in acinar cells consequent to reduced inflammatory infiltration. However, dissecting the direct role of HDAC in acinar proliferation during pancreatitis is precluded by the interference of the inflammatory reaction, thus we cannot exclude that MS-275 directly inhibits to some extent cell cycle progression in acinar cells. Increased HDAC activity is found in human pancreatic cancer cells [31, 32] and it is essential to support cell proliferation, NF-κB activation and EMT transition [33, 34]. However, since the role of HDACs in pancreatic cancer cells

cannot be directly translated to non-transformed acinar cells, additional non-tumor based experimental models are required to address the role of HDACs in acinar cell proliferation. In this regard, we observed HDAC up-regulation following pancreas resection, an intervention that triggers acinar proliferation with minimal levels of tissue inflammation (data not shown). As HDACs support hepatocyte proliferation following liver resection [35], the role of HDAC in acinar cell proliferation following pancreatectomy warrants further investigation.

Conclusions

Our data directly link the expression of class I HDACs to the pathophysiology of acute and chronic pancreatitis and underline the direct involvement of these enzymes in the development of the inflammatory response and in the de-differentiation of acinar cells, as summarized in Fig. 7E. Collectively, our data support the potential of therapeutic strategies based on inhibition of class I HDACs during pathological acute and chronic inflammatory insults of the pancreas. In addition, the up-regulation of HDAC isoforms observed during autoimmune pancreatitis prompts the exploration of the therapeutic effect of HDAC inhibition in this type of chronic pancreatitis, the treatment of which remains challenging due to the high frequency of relapses.

While the safety of several HDAC inhibitors, including MS-275, as anti-cancer treatments has been ascertained [36], additional clinical trials are needed to determine the correct therapeutic regimen in the setting of pancreatitis. In addition, given the existence of many HDAC isoforms, further studies with genetic knocked out models are required to understand their specificity or redundancy of functions, based on which selective isoform-selective inhibitors can be exploited to minimize toxicity.

All authors had access to the study data and had reviewed and approved the final manuscript.

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Author Contributions

The authors of this manuscript contributed in the study design, acquisition, analysis, interpretation of data, drafting and critical revision of the manuscript. MB performed experiments, generated and analyzed data, wrote the manuscript; ES, EM, RC, GMS, AZ performed experiments, generated and analyzed data; TR generated transgenic lines; CH, EQ,RG revised the manuscript; SS designed the study, wrote the manuscript. All authors approved the submitted version.

Conflict of interest

The authors declare no conflicts of interest.

Ethics approval

Animal experiments were approved by the cantonal veterinary office of Zurich in accordance with Swiss Federal animal regulations.

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FIGURE LEGENDS

Figure 1. HDACs are specifically up-regulated in the pancreas during acute and chronic pancreatitis. (**A**) Schematic representation of cerulein-induced acute pancreatitis. Grey boxes represent six intraperitoneal (i.p.) injections of 50 μg/kg cerulein (Cer) administered hourly over two consecutive days. Black triangles indicate the time of animal harvest, counting from the first cerulein injection. (**B**) qPCR of HDAC isoforms in the pancreas at the indicated time of acute pancreatitis. (**C**) Total HDAC activity in pancreata at the indicated time after induction of pancreatitis. (**D**) Immunostaining of acetylated proteins in the pancreas using an anti-acetylated lysine (AcK) antibody. (**E**) qPCR of HDAC isoforms in different organs 24h after induction of pancreatitis. (**F**) Schematic representation of cerulein-induced chronic pancreatitis. Grey boxes represent six cerulein injections administered on Monday, Wednesday and Friday. Animal harvests (black triangles) were performed on the following Monday after 2, 4 or 6 weeks of treatment. (**G**) qPCR of HDAC isoforms in the pancreas at the indicated time of chronic pancreatitis. (**H**) qPCR of HDAC isoforms in the pancreas of 12 month old wild type (WT) and lymphotoxin αβ–overexpressing (Tg+) mice with autoimmune pancreatitis. Results are average ± SEM (n=5), **P* < 0.05. Scale bars: 50 μm.

Figure 2. MS-275 treatment does not limit the initial acinar cell damage but reduces the infiltration of inflammatory cells following acute pancreatitis. (**A**) Schematic representation of MS-275 regimen during induction of pancreatitis. Grey box represents the cerulein (Cer) treatment administered on two consecutive days. MS-275 was injected daily i.p. at 20 mg/kg (MS) or 40 mg/kg (2xMS). (**B**) HDAC activity in the pancreas following pancreatitis induction in the presence of MS-275. (**C**) Serum levels of amylase. (**D**) Serum levels of glutamic-pyruvic transaminase (GPT). (**E**) Quantification of pancreatic trypsinogen activation. (**F**) Quantification of TUNEL-positive apoptotic acinar cells. Right panel, representative image of stained cells (arrows). (**G**) qPCR of chemokine expression in the pancreas. (**H**) Quantification of PU.1-positive cells infiltrating the pancreas at the indicated time after induction of pancreatitis. Right panel, representative image of stained cells (arrows). (**I**) qPCR of the macrophage marker F4/80. (**J**) qPCR of macrophage subtype markers in pancreata. (**K**) qPCR of the T cell marker CD3 in pancreata. (**L**) Quantification of CD3-positive T cells in pancreata. Results are average ± SEM (n=5), **P* < 0.05. Scale bars: 50 μm.

Figure 3. Preventive and therapeutic administration of MS-275 reduces the infiltration of inflammatory cells following chronic pancreatitis. (**A**) Schematic representation of two weeks of preventive (MS+Cer, red box) and therapeutic (Cer+MS, orange box) MS-275 regimens during induction of chronic pancreatitis. Grey box represents the cerulein (Cer) treatment administered on alternate days over four weeks. MS-275 was injected i.p. on alternate days at 20mg/kg. (**B**) Haematoxylin and eosin (H&E) staining of pancreata four weeks following induction of chronic pancreatitis in the presence of MS-275. (**C**) Quantification of PU.1-positive cells infiltrating the pancreas. (**D**) Immunostaining of F4/80-positive macrophages infiltrating the pancreas. (**E**) qPCR of activated macrophages, macrophage subtypes, CD3-positive T cells and pro-inflammatory markers in pancreata. (**F**) Immunostaining and quantification of CD3-positive T cells infiltrating the pancreas. Results are average ± SEM (n=5), **P* < 0.05. Scale bars: 50 μm.

Figure 4. MS-275 treatment alters the activation of macrophages. (**A**) HDAC expression in RAW264.7 macrophages upon activation with 10 ng/mL LPS for 6h. (**B**) HDAC activity in LPS-treated RAW264.7 macrophages in the presence of 1 μM MS-275. (**C**) Cell viability of LPS-treated RAW264.7 macrophages in the presence of 1 μM MS-275. (**D**) Diameter increase of LPS-treated RAW264.7 macrophages in the presence of 1 μM MS-275. Right panels,

representative micrographs of treated cells. (**E**) qPCR of inflammatory markers in LPS-activated RAW264.7 macrophages incubated with 1 μM MS-275. (**F**) qPCR of inflammatory markers in LPS-activated primary mouse macrophages incubated with 1 μM MS-275. Results are average ± SEM (n=3), *P < 0.05. Scale bars: 20 μm.

Figure 5. MS-275 treatment reduces the number of proliferating cells in the pancreas during acute and chronic pancreatitis. (**A**) Quantification of Ki67-positive and pH3-positive acinar cells at the indicated time of pancreatitis induction. (**B**) Examples of Ki67 and pH3 immunostainings. Acinar cells (arrows) were identified by large and round nuclei located inside the acinar periphery, while interstitial cells (arrowheads) had smaller and elongated nuclei present outside the pancreatic acini. (**C**) Quantification of Ki67-positive and pH3-positive interstitial cells. (**D**) qPCR of cyclin A and B expression in the pancreas. (**E**) qPCR of p21 expression in the pancreas. (**F**) qPCR of inflammatory markers in p21 deficient ($p21^{-/-}$) mice 72h after induction of pancreatitis in the presence of MS-275. (**G**) Quantification of Ki67-positive and pH3-positive acinar and interstitial cells in $p21^{-/-}$ mice 72h after induction of pancreatitis in the presence of MS-275. (**H**) qPCR of cyclins (Cy), CDK inhibitors and p53 in $p21^{-/-}$ mice 72h after induction of pancreatitis in the presence of MS-275. (**I**) Quantification of Ki67-positive acinar and interstitial cells after four weeks of chronic pancreatitis following preventive (MS+Cer) and therapeutic (Cer+MS) MS-275 regimens. (**J**) qPCR of cyclins after four weeks of chronic pancreatitis with preventive and therapeutic MS-275 regimens. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μm.

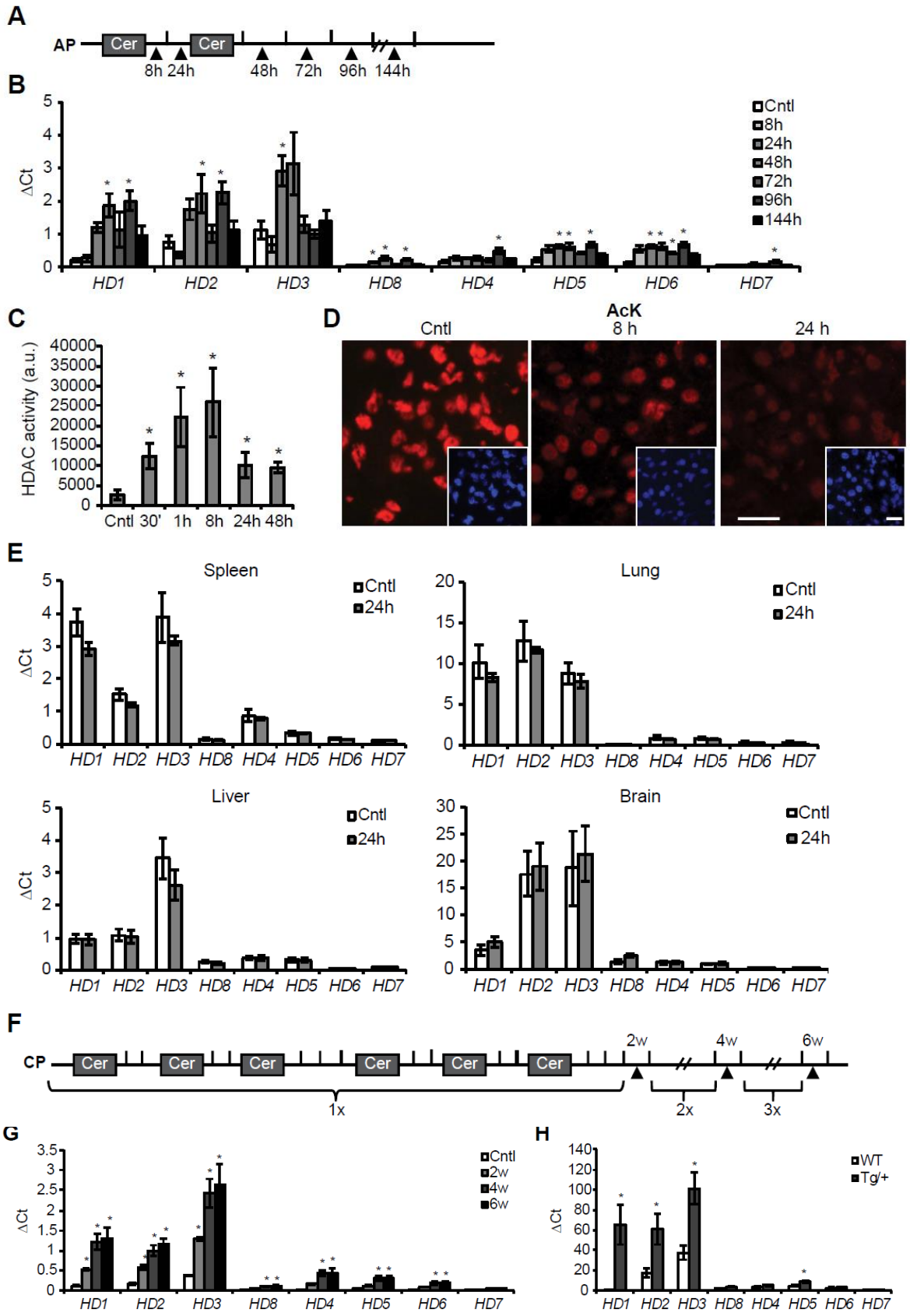
Figure 6. MS-275 treatment ameliorates the outcome of acute and chronic pancreatitis. (**A**) Quantification of γH2AX-positive acinar cells, indicative of DNA damage, in wild-type C57BL/6 (BL/6) and p21 deficient ($p21^{-/-}$) mice following acute pancreatitis in the presence of MS-275. Right panel, representative micrograph of stained acinar cells (arrows). I, islet. (**B**) Quantification of ADMs in BL/6 and $p21^{-/-}$ mice 72h after induction of pancreatitis in the presence of MS-275. Right panel, ADM areas (asterisks) are quantified after tissue immunostaining with amylase and are expressed as % of the total pancreatic area. (**C**) Immunostaining 48 hours after induction of pancreatitis showing Sox9-positive acinar (A) and centro-acinar (CA) cells, ductules (D) and ADM (asterisks). (**D**) Quantification of Sox9-positive acinar cells during induction of pancreatitis in the presence of MS-275. (**E**) Quantification of non-acinar tissue after four weeks of chronic pancreatitis following preventive (MS+Cer) and therapeutic (Cer+MS) MS-275 regimens. (**F**) qPCR of progenitor-like markers after four weeks of chronic pancreatitis following preventive and therapeutic MS-275 regimens. (**G**) qPCR of EGF receptor (EGFR) after four weeks of chronic pancreatitis following preventive and therapeutic MS-275 regimens. (**H**) Western blotting quantification of EGFR expression after four weeks of chronic pancreatitis following preventive and therapeutic MS-275 regimens. Band intensity values were normalized using tubulin as a loading control. (**I**) Immunostaining of phosphorylated EGFR (p-EGFR) showing the active form of the receptor after four weeks of chronic pancreatitis following preventive and therapeutic MS-275 regimens. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μm.

Figure 7. MS-275 treatment reduces acinar de-differentiation into ADM. (**A**) qPCR of HDAC isoforms in acinar explants from BL/6 mice 5 days after TGFα-induced de-differentiation. (**B**) Quantification of ADM in acinar explants after 5 days of TGFα stimulation in the presence of MS-275 (MS) at the indicated concentrations. Right panels, examples of TGFα-induced ADMs (arrows). (**C**) qPCR of EGF receptor (EGFR) and progenitor like-genes in acinar explants after 5 days of TGFα stimulation. (**D**) Metabolic activity of acinar explants visualized by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into insoluble

chromogenic formazan blue crystals. (**E**) Schematic representation of the effects exerted by class I HDAC inhibition with MS-275 during acute and chronic pancreatitis. Solid lines, direct effects; dashed lines, potentially indirect effects. Results are average ± SEM (n=4), *$P < 0.05$. Scale bars: 50 μm.

Figures:

Figure 1



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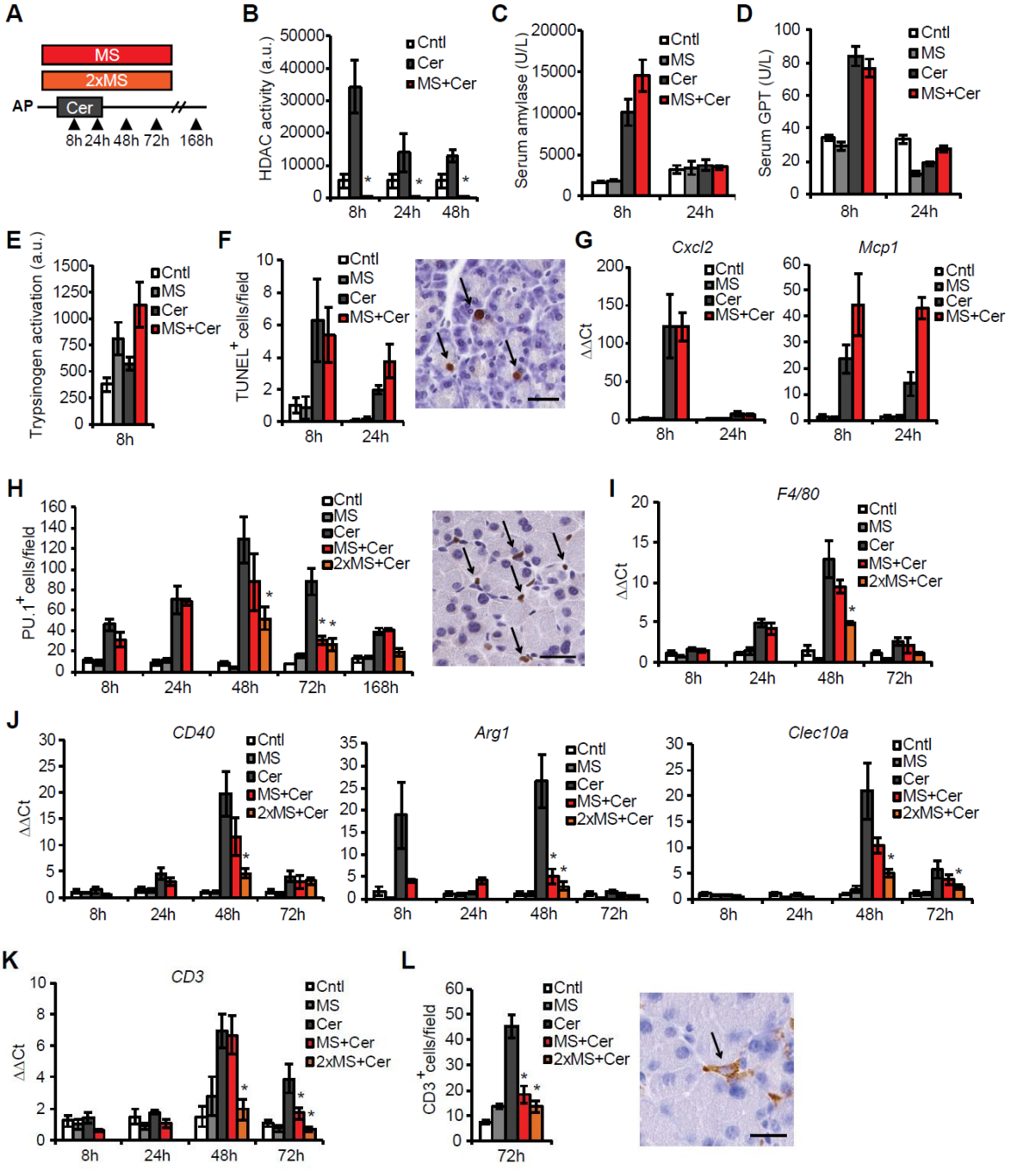
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Figure 3

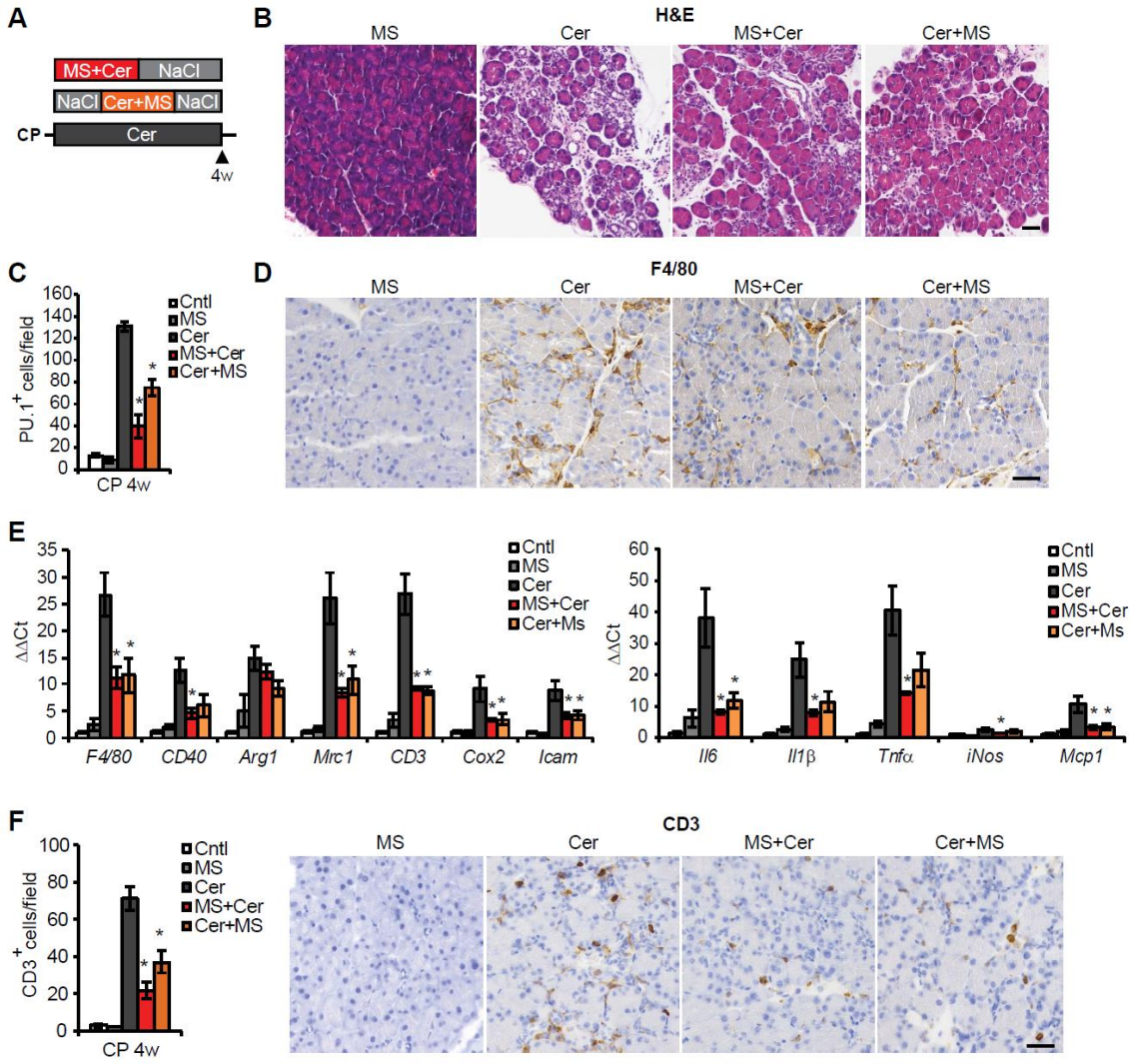
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Figure 4

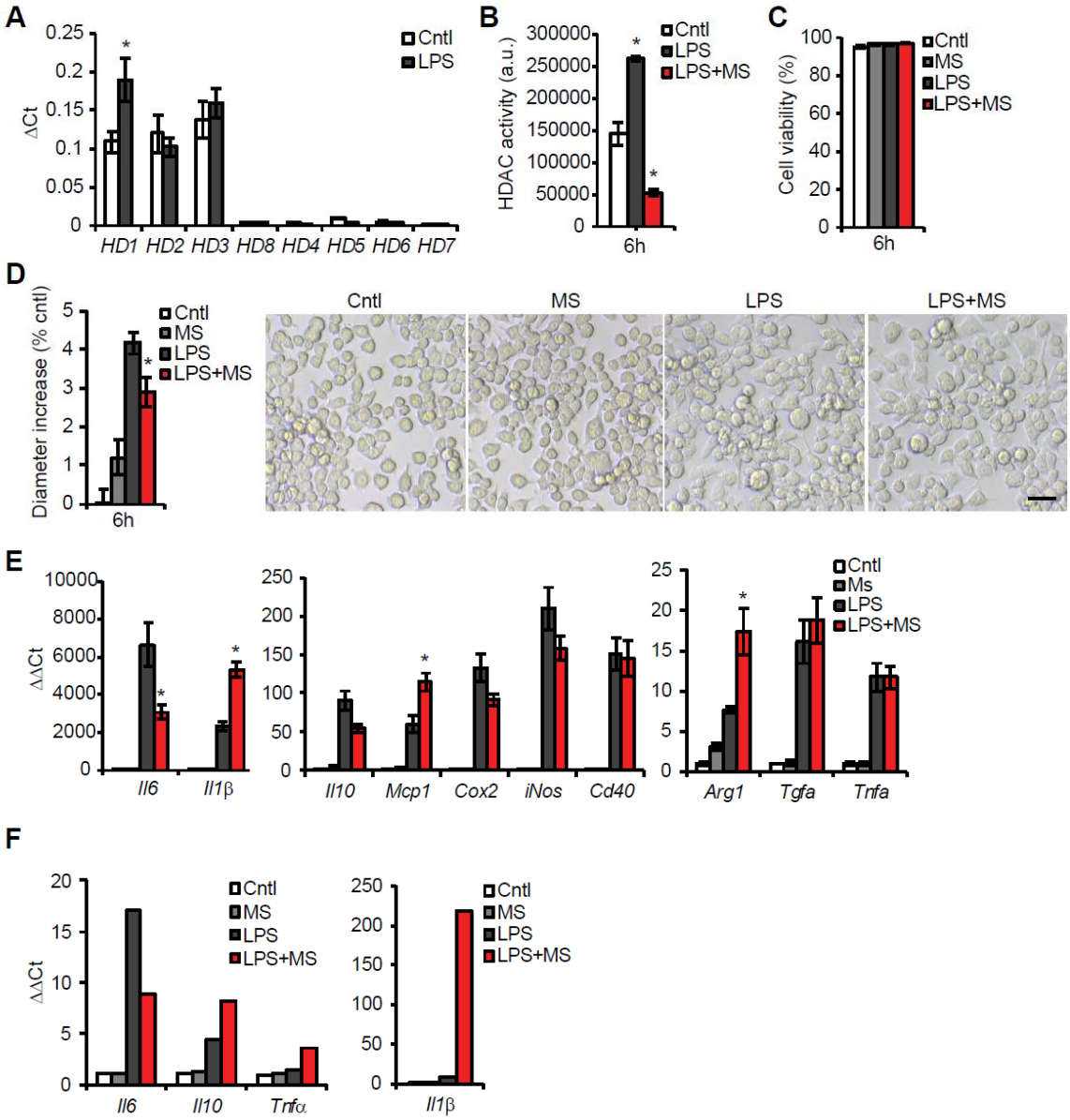
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Figure 5

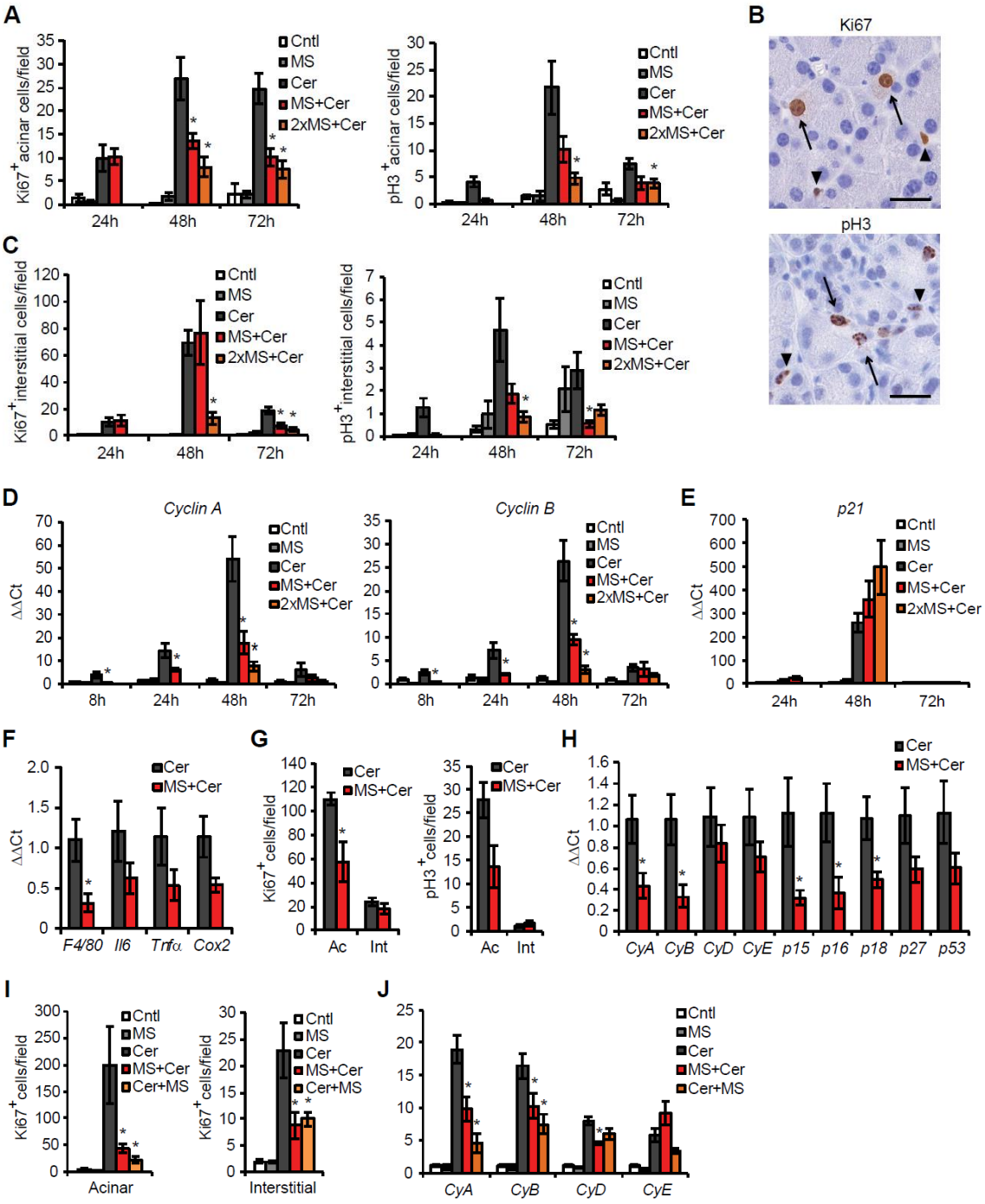
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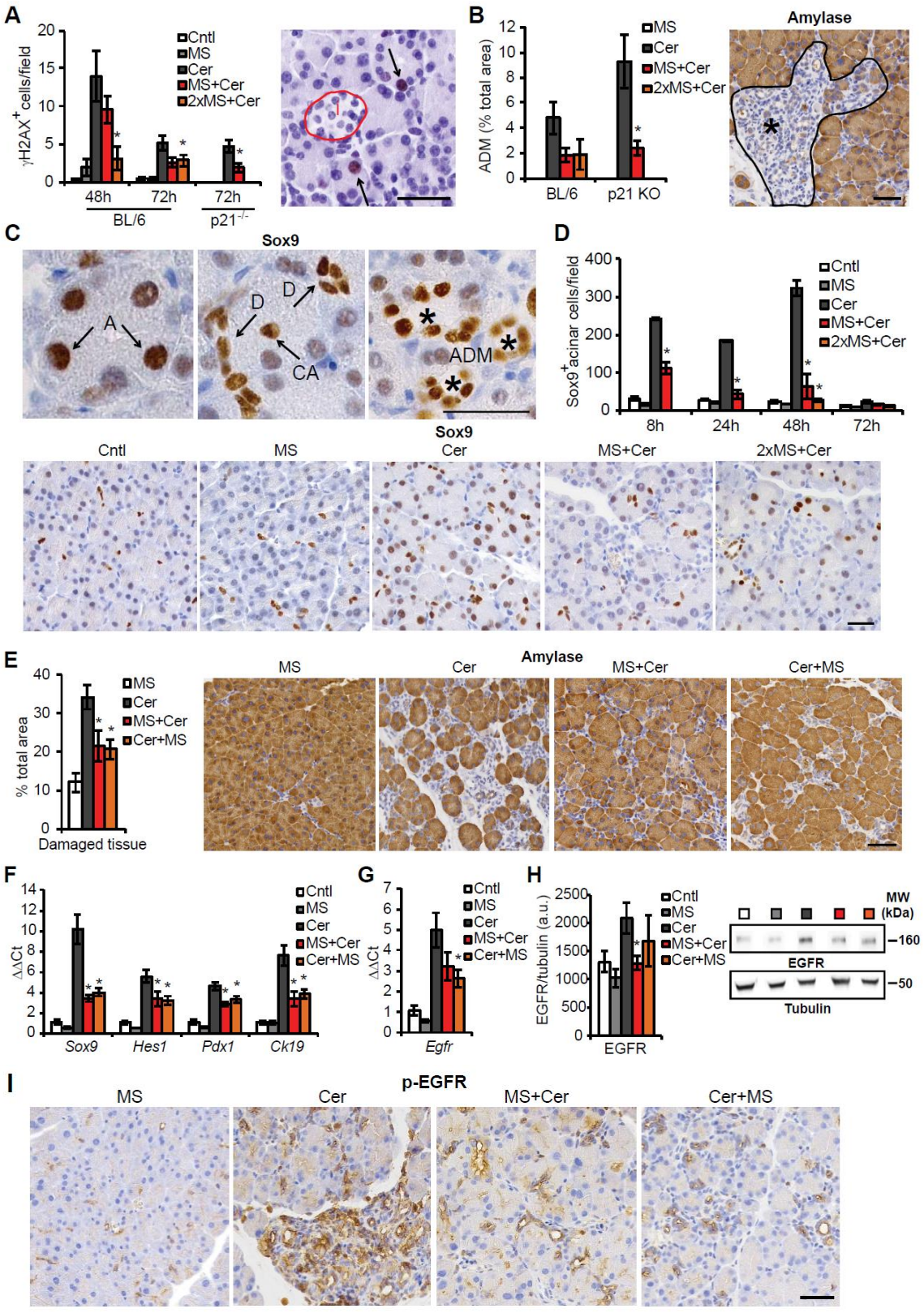
Figure 6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Cer+MS
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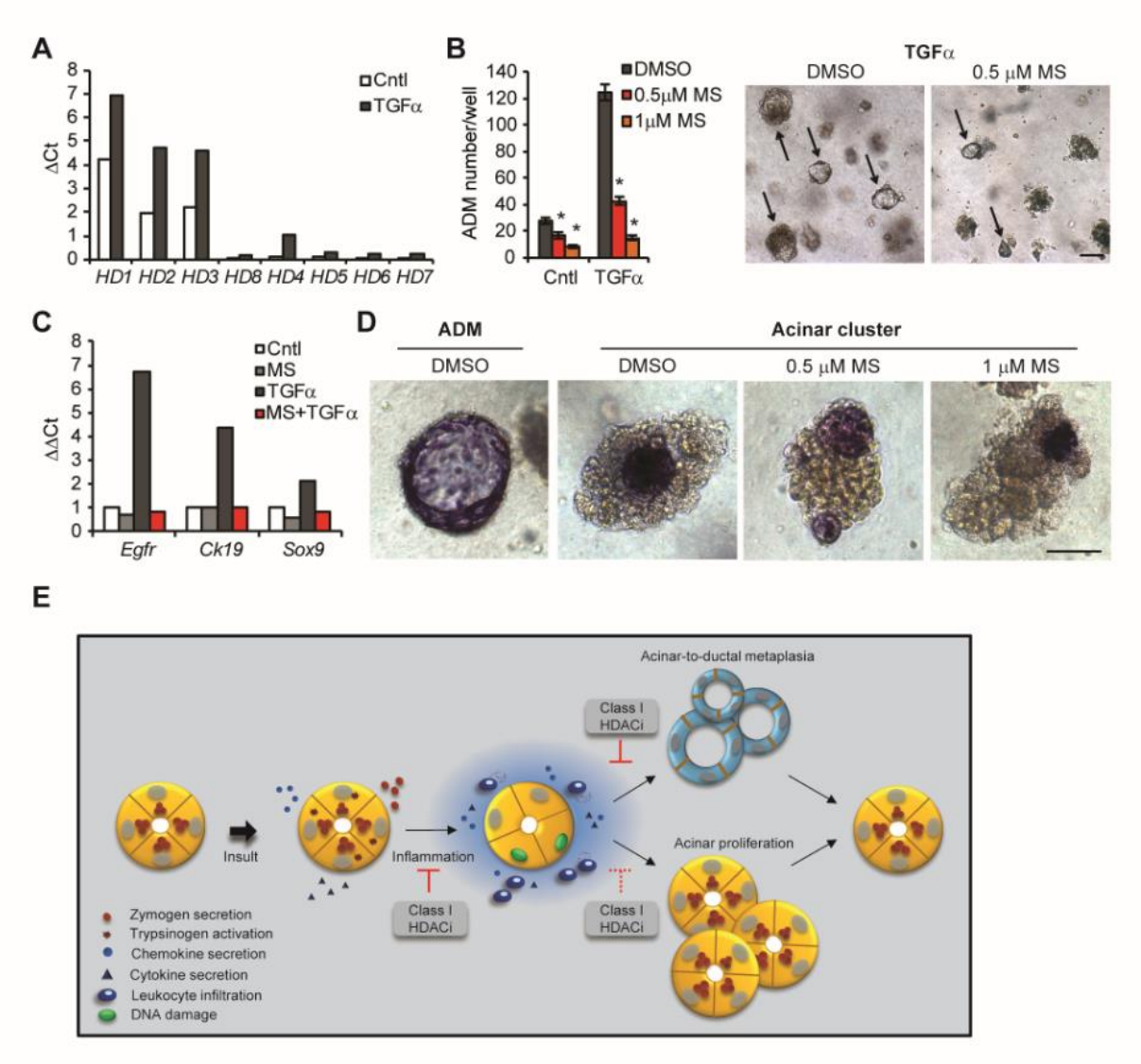
MS
Cer
MS+Cer
Cer+MS

Figure 7


A
ΔCt
Cntl
TGFα
HD1 HD2 HD3 HD8 HD4 HD5 HD6 HD7
B
ADM number/well
DMSO
0.5μM MS
1μM MS
Cntl TGFα
TGFα
DMSO
0.5 μM MS
C
ΔΔCt
Cntl
MS
TGFα
MS+TGFα
Egfr Ck19 Sox9
D
ADM
Acinar cluster
DMSO
DMSO
0.5 μM MS
1 μM MS
E
Acinar-to-ductal metaplasia
Class I HDACi
Insult
Inflammation
Acinar proliferation
Class I HDACi
Class I HDACi
Zymogen secretion
Trypsinogen activation
Chemokine secretion
Cytokine secretion
Leukocyte infiltration
DNA damage

SUPPLEMENTARY FIGURE LEGENDS

Supplementary figure 1. (**A**) Quantification of PU.1-positive cells infiltrating the pancreas at the indicated time after induction of acute pancreatitis. (**B**) Immunostaining of PU.1-positive cells in the pancreas. Right panel, example of positive cells (arrows). (**C**) Immunostaining of coronin-1-positive cells in the pancreas. Right panel, example of positive cells (arrows). Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

Supplementary figure 2. (**A**) qPCR of HDAC isoforms in isolated acinar cells isolated from pancreata 24h after induction of pancreatitis. (**B**) qPCR of HDAC isoforms in WT $Tph1^{+/+}$ and $Tph1^{-/-}$ pancreata 24h after induction of pancreatitis. (**C**) qPCR of HDAC isoforms in WT and Tgf-$\beta RII^{fl/fl}$ pancreata 24h after induction of pancreatitis. Results are average ± SEM (n=5), *P < 0.05.

Supplementary figure 3. (**A**) qPCR of inflammatory markers at the indicated time after induction of chronic pancreatitis.(**B**) Haematoxylin and eosin (H&E) staining of pancreata at the indicated time after induction of chronic pancreatitis. (**C**) HDAC activity in the pancreas following 4 weeks of chronic pancreatitis. (**D**) qPCR of inflammatory markers in 12 month old wild type (WT) and lymphotoxin αβ–overexpressing mice (Tg+), which spontaneously develop autoimmune pancreatitis. (E) Haematoxylin and eosin (H&E) staining of pancreata from 12 month old WT and Tg+ mice. TLO, tertiary lymphoid organ, I, islet. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

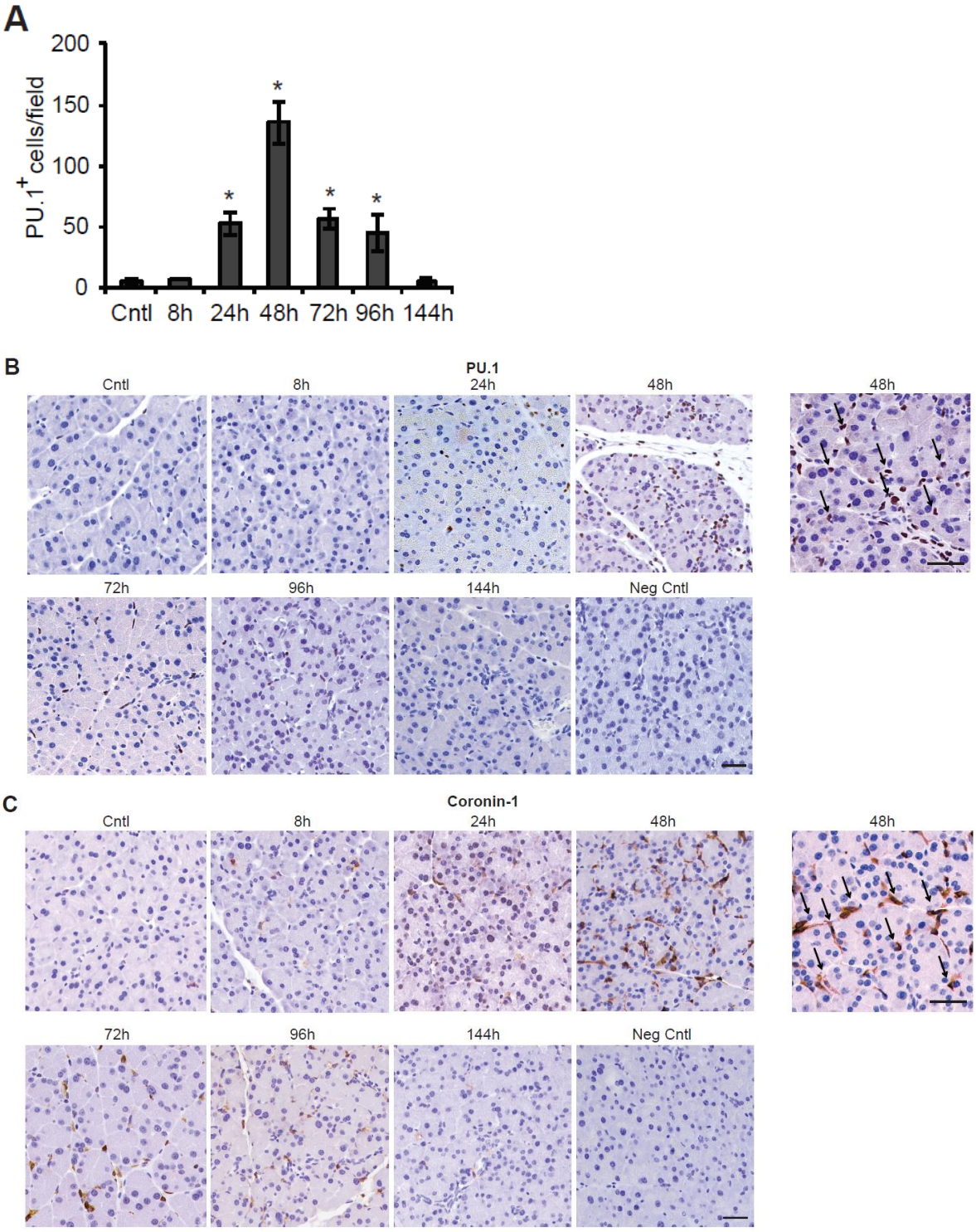
Supplementary figure 4. (**A**) Haematoxylin and eosin (H&E) staining of pancreata at the indicated time upon treatment with 20 mg/kg MS-275 or vehicle control. (**B**) Quantification of serum amylase at the indicated time following MS-275 treatment. (**C**) Quantification of serum GPT at the indicated time following MS-275 treatment. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

Supplementary figure 5. qPCR of HDAC isoforms in pancreata at the indicated time after induction of pancreatitis in the presence of MS-275. Results are average ± SEM (n=5), *P < 0.05.

Supplementary figure 6. (**A**) Coronin-1 staining of pancreata 48h after induction of pancreatitis in the presence of MS-275. (**B**) qPCR of inflammatory markers in pancreata at the indicated time after induction of pancreatitis in the presence of MS-275. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

Supplementary figure 7. qPCR of cyclins (**A**) and cell cycle regulators (**B**) at the indicated time after induction of pancreatitis in the presence of MS-275. Results are average ± SEM (n=5), *P < 0.05.

Supplementary figure 1



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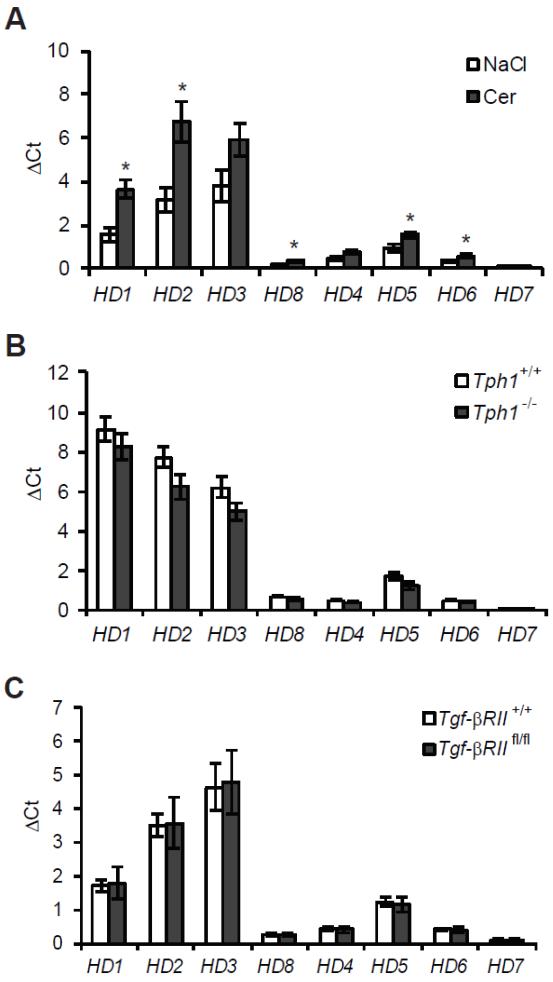

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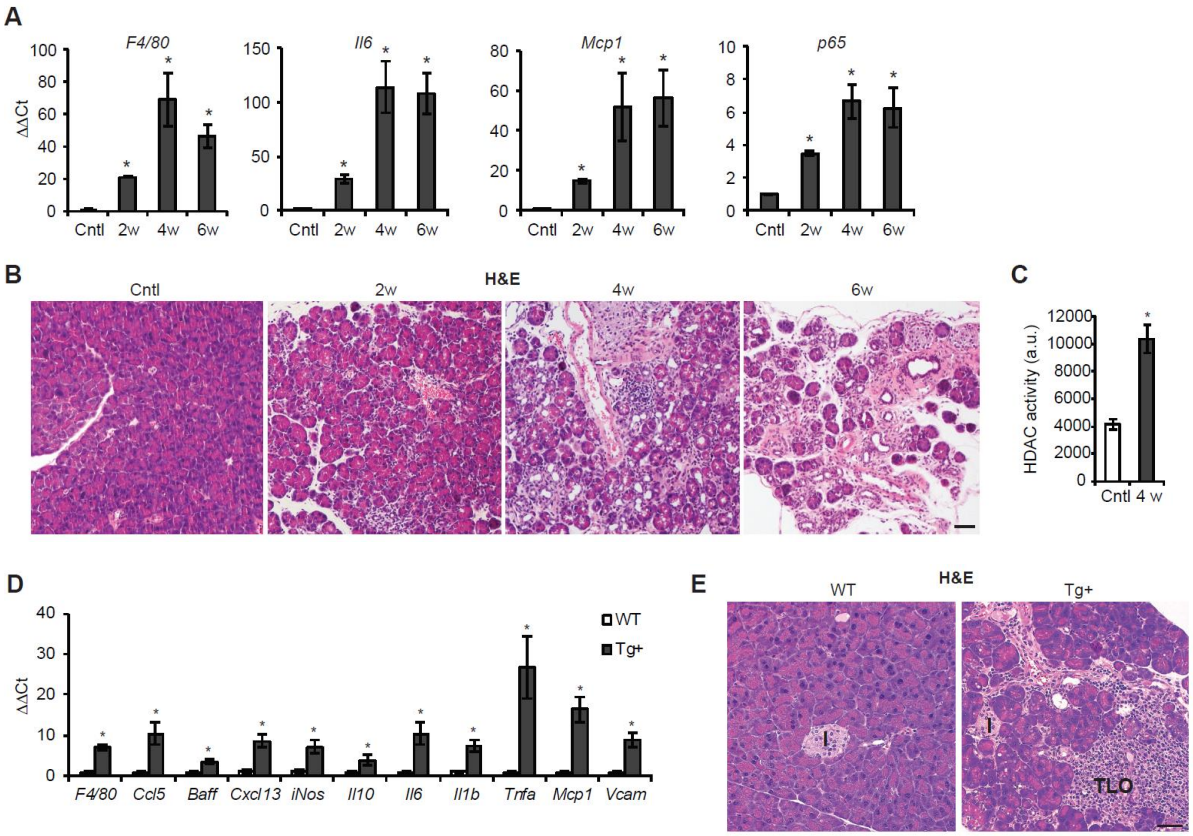

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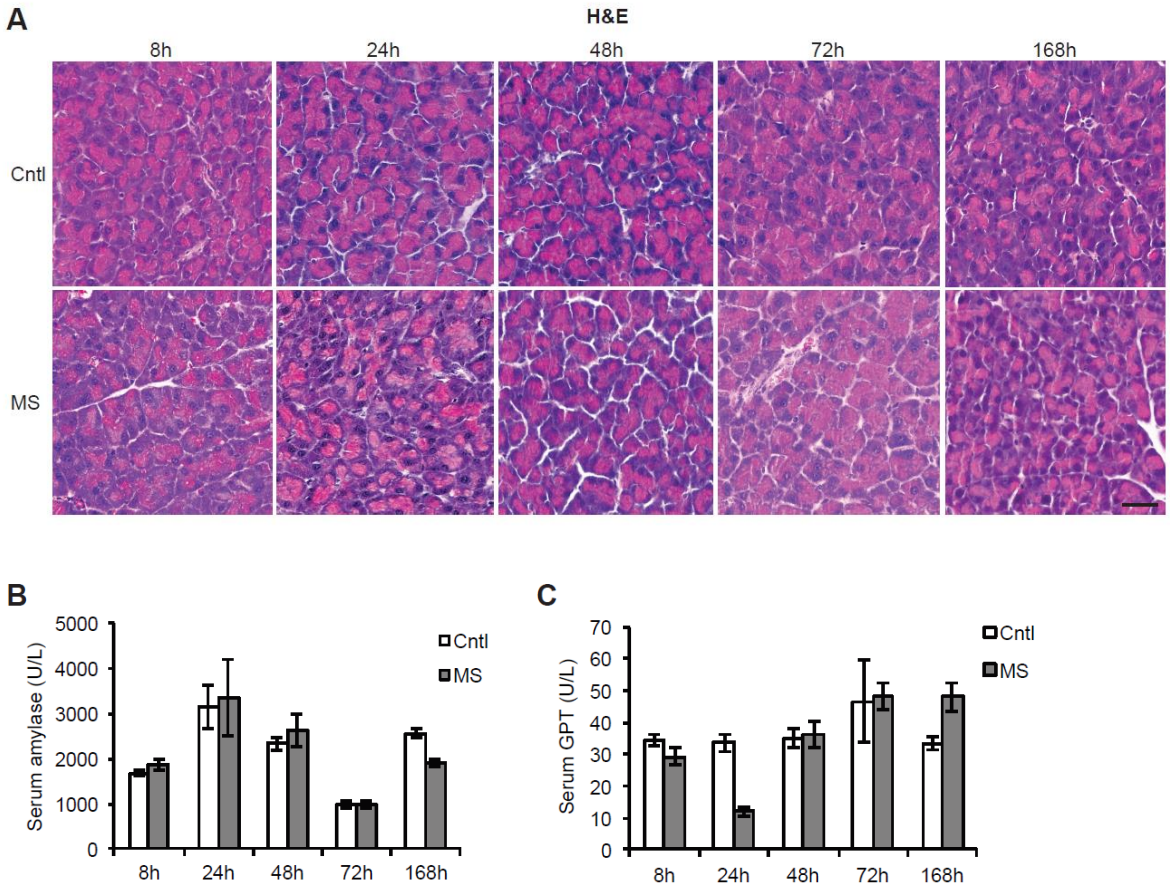
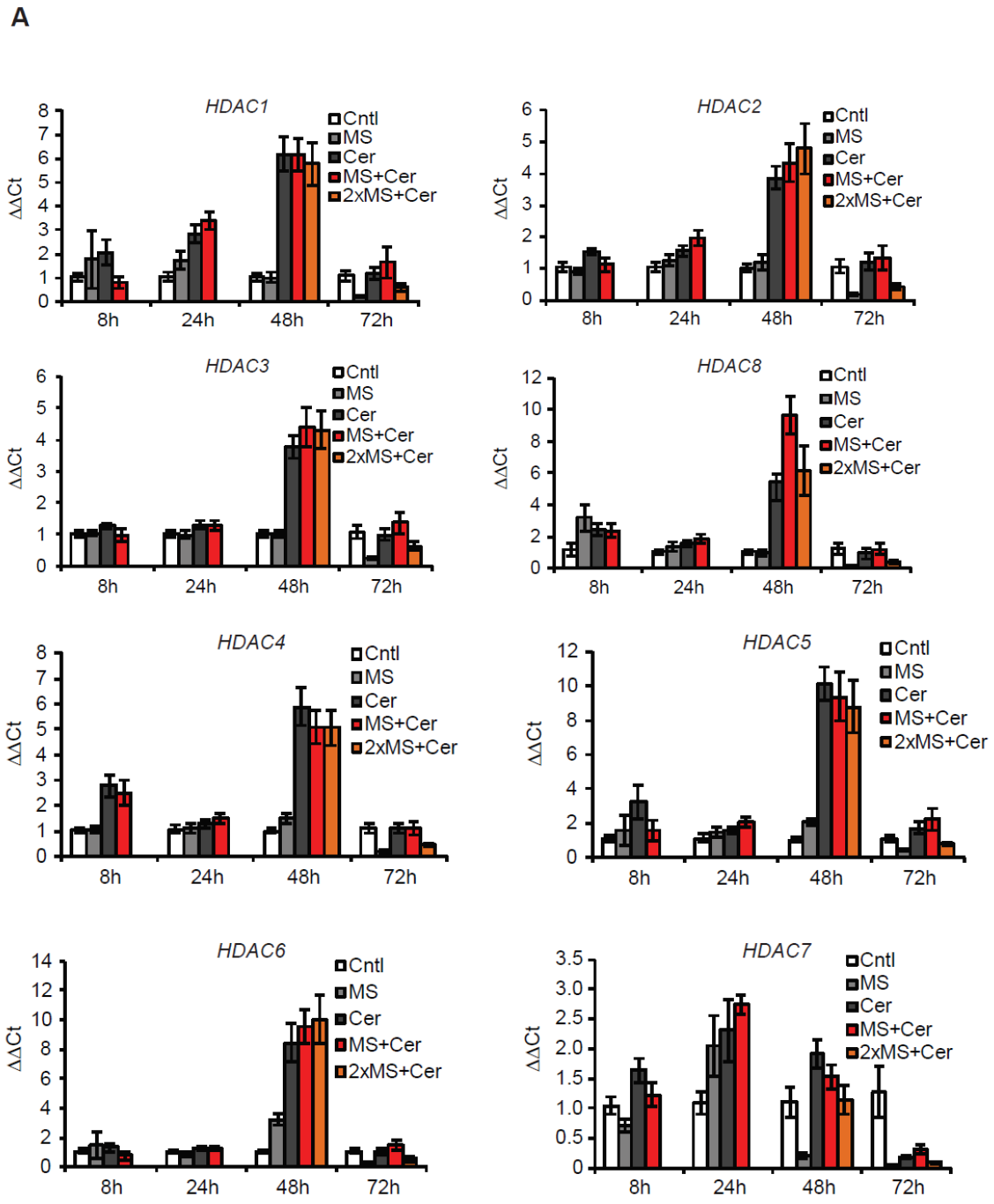
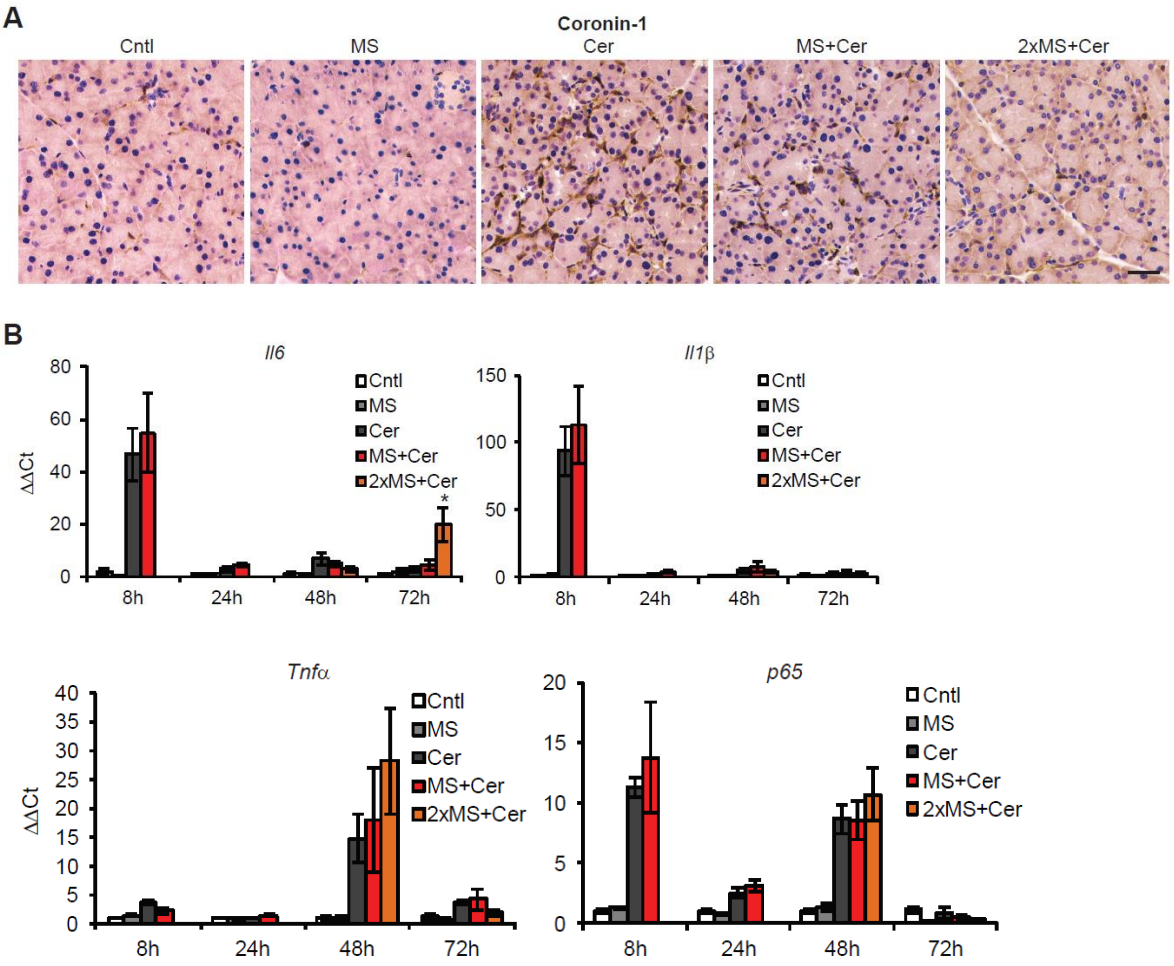


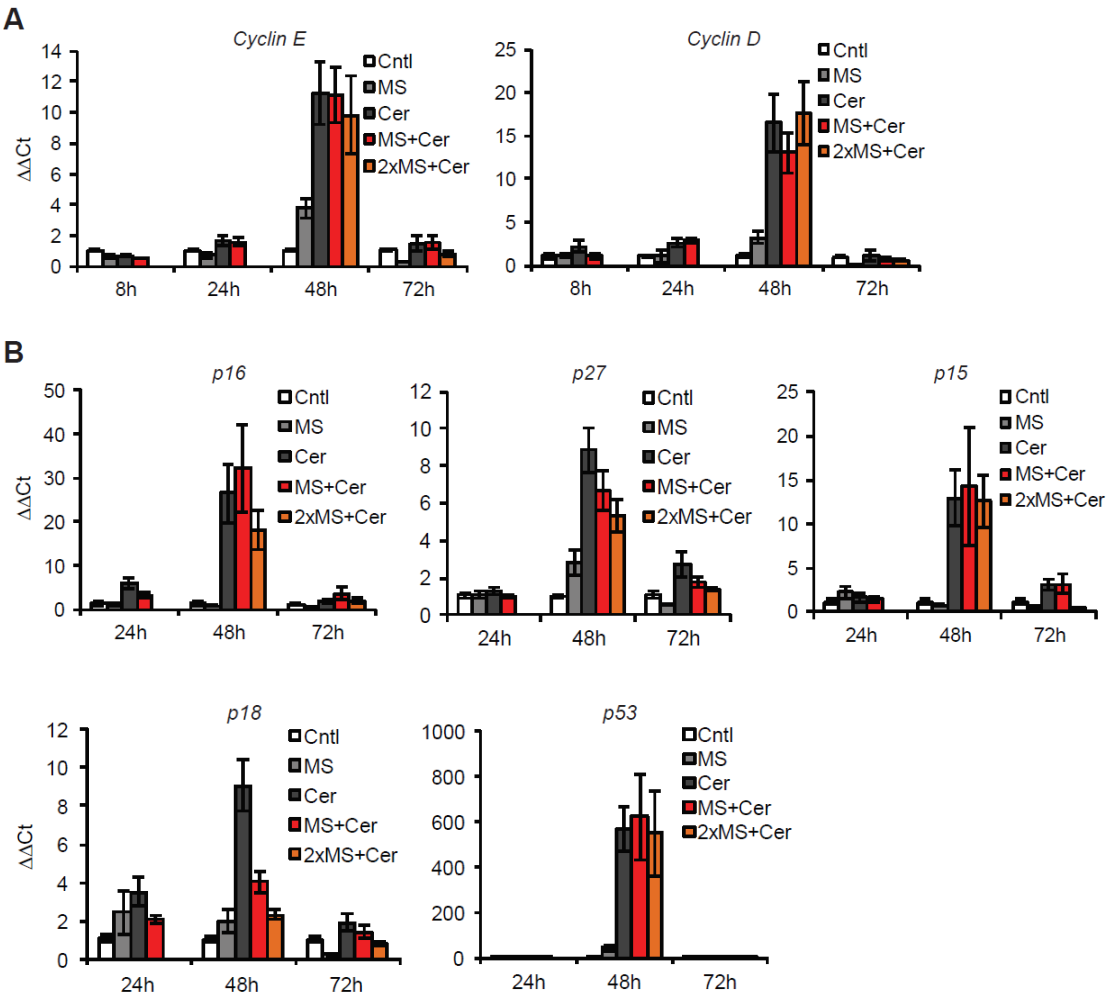
Supplementary figure 5



Supplementary figure 6



Supplementary figure 7



Manuscript B

Inhibition of class I histone deacetylases abrogates TGFβ expression, pancreatic stellate cell activation and fibrosis during chronic pancreatitis

Marta Bombardo, Enrica Saponara, Ermanno Malagola, Rong Chen, Rolf Graf and Sabrina Sonda.

To be submitted in 2017

Inhibition of class I histone deacetylases abrogates TGFβ expression, pancreatic stellate cell activation and fibrosis during chronic pancreatitis

Short title: MS-275 ameliorates the outcome of chronic pancreatitis

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Conflicts of interest The authors disclose no conflicts.

# Equal contribution

Word count 2650

ABSTRACT

Pancreatic fibrosis is the hallmark of chronic pancreatitis, a highly debilitating disease for which there is currently no cure. The key event at the basis of pancreatic fibrosis is the deposition of extracellular matrix proteins by activated pancreatic stellate cells (PSC). Transforming growth factor β (TGFβ) is a potent pro-fibrotic factor in the pancreas as it promotes the activation of PSC. Thus pharmacologic interventions that effectively reduce TGFβ expression harbor considerable therapeutic potential in the treatment of chronic pancreatitis. In this study we investigated the molecular link between TGFβ expression and the activity of the epigenetic modifiers histone deacetylases (HDACs). To this aim, chronic pancreatitis was induced in C57BL/6 mice with serial injection of cerulein and the selective class I HDAC inhibitor MS-275 was administered *in vivo* in a preventive and therapeutic manner. Both MS-275 regimens potently reduced deposition of extracellular matrix and development of fibrosis in the pancreas after four weeks of chronic pancreatitis. Reduced pancreatic fibrosis was concomitant to reduced PSC activation and lower expression of pancreatic TGFβ. In search of the cell types targeted by the inhibitor, we found that MS-275 treatment hampered the expression of TGFβ in isolated primary acinar cells.
Our study demonstrates that MS-275 is an effective anti-fibrotic agent in the context of experimental chronic pancreatitis and thus may constitute a valid therapeutic intervention for this severe disease. In addition, it highlights the concept that TGFβ expressed by acinar cells may constitute a key factor in paracrine activation of PSC.

INTRODUCTION

Chronic pancreatitis is defined as a progressive inflammation of the pancreas. However, development of organ fibrosis is at the core of the disease pathophysiology. This progressive condition is characterized by irregular sclerosis with focal, segmental, or diffuse destruction of the parenchyma. As a consequence, gradual loss of exocrine and endocrine cellular components leads to pancreatic insufficiency and eventually diabetes, which are associated with considerable morbidity and reduction of the life quality and expectancy of patients (reviewed in (1,2)).
Fibrosis is characterized by excessive production and deposition of extracellular matrix (ECM) components in the pancreatic parenchima. ECM proteins are mainly produced by pancreatic stellate cells (PSC) resident in the organ. In response to organ injury, released pro-fibrogenic factors activate PCS, a process characterized by phenotypical cell alteration, proliferation and ECM protein synthesis.
Despite the research advances accomplished so far, the complex cellular and signaling mechanisms that drive the fibrotic process are not yet completely elucidated. This limited knowledge explains why therapeutic approaches that effectively counteract the development of organ fibrosis are not currently available and management of chronic pancreatitis remains a clinical challenge.
In this study we evaluated whether administration of MS-275, a selective inhibitor of the epigenetic modifiers class I histone deacetylases (HDACs), counteracts the development of pancreatic fibrosis using the widespread murine model of cerulein-induced chronic pancreatitis. The rationale for this approach is threefold: i) epigenetic mechanisms are likely to orchestrate the substantial gene regulation observed in the development of fibrosis, ii) HDACs are critical epigenetic regulators and we found that expression of class I HDACs is significantly up-regulated during the course of chronic pancreatitis (Manuscript A), iii) pharmacological inhibitors of HDAC activity, while originally developed as anti-cancer agents, are currently being investigated for their anti-fibrotic properties in different fibrotic diseases (recently reviewed in (3-6)).

MATERIALS AND METHODS

Animal experiments

All animal treatments were performed in accordance with Swiss Federal animal regulations and approved by the cantonal veterinary office of Zurich. 8 weeks wild-type C57BL/6 mice (Envigo Laboratories, Horst, The Netherlands) were used. Animals were kept under standardized conditions under 12:12 light/dark cycles, with food and water available *ad libitum.*

Chronic pancreatitis was induced via six intraperitoneal (i.p.) injections of cerulein (50µg/kg) administered hourly every second day for up to 6 weeks. Control animals received 0.9% NaCl injections. MS-275 (Selleckchem, Houston, USA) was injected i.p. at 20mg/kg every second day for two weeks starting concomitantly (preventive regimen) or one week after the beginning of cerulein injections (therapeutic regimen). Controls animals received DMSO injections. I.p. injections were alternated daily between left and right sides of the abdomen.

Mammalian cell cultures

Acinar cells were isolated from 6 week old Wistar rats from Charles River Deutschland (CRL). Acini were pre-incubated with 1 µM MS-275 for 30 minutes and stimulated with 0.1 nM cerulein for 30 minutes in the presence of 1 µM MS-275. At the end of the treatment, cells were lysed in the plates for RNA extraction and real-time PCR analysis.

Immunohistochemistry

Pancreas specimens were embedded in paraffin for histological analyses, as previously described (7). Hematoxylin and eosin and Masson's trichrome staining were performed according to routine procedures. α-SMA staining was performed with a mouse anti-α-SMA primary antibody (Dako, Glostrup, Denmark). Secondary antibodies used in this study were biotinylated goat anti-IgG (H + L), included in the Vectastain® ABC Kit (PK-4001).

Microscopy analyses were performed on a wide-field Nikon Eclipse Ti (Amsterdam, The Netherlands). Quantification of labelled cells was performed in at least 10 randomly selected high-power fields (×200) per slide using the NIS Elements BR Analysis and Cell^P analysis software.

Western blotting

20 mg of pancreatic tissue was homogenized in RIPA buffer containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by a Bradford protein assay (BioRad, Hercules, CA, USA). 20 µg of proteins were resolved by SDS-PAGE electrophoresis and blotted onto nitrocellulose membranes using a V3 Western Workflow system (BioRAd, Hercules, CA, USA) according to the manufacturer's protocols.

Membranes were incubated with primary antibodies overnight at 4°C. Primary antibodies used in this study were: mouse anti-α-SMA (Dako, Glostrup, Denmark); rabbit anti α-Tubulin (#ab52894, Abcam); rabbit anti-phospho-Smad3 (Ser423/425) (Cell Signaling, Danvers, MA, USA); rabbit anti-GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA).

Nuclear protein extract and HDAC activity

Nuclear proteins were extracted from 20 mg of pancreatic tissue with the EpiQuik™ Nuclear Extraction Kit (Epigentek Group Inc, Mountain View, CA) and HDAC activity was measured in the nuclear extracts with the fluorimetric EpiQuik HDAC Activity/Inhibition Assay Kit (Epigentek Group Inc. Mountain View, CA), following the manufacturer's instructions.

Transcript analyses

Total RNA was extracted from pancreatic tissue and acinar explants as described previously (8) and reverse-transcribed with qScript™ cDNA SuperMix (Quanta Biosciences, Beverly, MA, USA). Gene expression was measured by real-time PCR on a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using Taqman probes (Applied Biosystems, Carlsbad, CA, USA). Transcript levels were normalized using 18S RNA as a reference

and expressed as ΔΔCt relative to the value of control animals.

Statistical analyses

Groups of 5 animals were tested for each experiment. The data are expressed as means ± SEM. The statistical significance of differences in the means of experimental groups was determined using an unpaired, two-tailed Student's *t* test or one-way analysis of variance followed by Dunnett's post-hoc test (GraphPad Prism 4.0c; GraphPad Software, Inc.) and a probability value <0.05 was considered statistically significant.

RESULTS

Development of fibrotic response during chronic pancreatitis correlates with increased levels of HDAC expression.

To investigate the role of HDAC in the development of pancreatic fibrosis following induction of chronic pancreatitis, we first performed a time-course analysis following induction of the disease to determine the kinetic of the fibrotic response. Histological evaluation of mice harvested after two, four and six weeks of cerulein treatment revealed a progressive damage of pancreatic parenchima and cell infiltration (Fig. 1A) and pronounced extracellular matrix (ECM) deposition (Fig. 1B). Further quantification of fibrotic parameters showed that increased expression of collagen reached a plateau level after 4 weeks of pancreatitis (Fig. 1C). Development of pancreatic fibrosis is mediated by activated pancreatic stellate cells (PSCs), which constitute the predominant source of ECM proteins, including collagens and fibronectin. Activation of PSCs, detected by α-smooth muscle actin (α-SMA) expression, reached a plateau after 4 weeks of pancreatitis (Fig. 1D), thus mirroring the kinetic of collagen expression. Expression of pro-fibrotic TGFβ isoforms, the main drivers of PSC activation, and TGFβ receptor also increased in a similar pattern (Fig. 1E), suggesting a concerted action of PCS activation and signalling mediators to promote the development of organ fibrosis. In addition, levels of pro-inflammatory factors followed a similar kinetic of expression in the pancreas (Fig. S1). Based on these results showing maximal levels of fibrosis after 4 weeks of pancreatitis, we chose to focus on this time point for our further analyses. We recently showed that gene expression levels of class I HDACs were upregulated during the course of chronic pancreatitis (Manuscript A). This was further confirmed by increased HDAC enzymatic activity (Fig. 1F) in pancreatic nuclear proteins.

Inhibition of class I HDAC with MS-275 reduces the development of fibrosis following induction of chronic pancreatitis.

To test whether class I HDAC up-regulation was functionally linked to the development of fibrosis, we treated mice with the selective class I inhibitor MS-275 (Entinostat). To this aim we administered the inhibitor for two weeks in a preventive manner, namely starting concomitantly with the first cerulein injection. Alternatively, we administered the inhibitor in a therapeutic manner, starting after one week of pancreatitis. Regimen schemes are depicted in Fig. 2A. Animals were harvested after four weeks of chronic pancreatitis. Histological examination revealed that both modalities of MS-275 treatment resulted in better preservation of pancreatic parenchima and limited fibrosis (Fig. 2B). This was further confirmed by reduced collagen deposition (Fig. 2C) and reduced collagen expression following MS-275 administration (Fig. 2D). As collagen deposition is mainly achieved by activated PSCs, we next evaluated whether PSC activation was reduced in the presence of MS-275. Gene expression of α-SMA, a key hallmark of PSC activation, was lower upon MS-275 treatment (Fig. 3A). Reduced expression of α-SMA was further confirmed at protein level by western blotting (Fig. 3B). These data revealed that both preventive and therapeutic regimens of MS-275 were effective in reducing PSC activation and consequently limiting the development of

fibrotic processes upon induction of chronic pancreatitis.

Inhibition of class I HDAC with MS-275 reduces TGFβ expression following induction of chronic pancreatitis.

Activation of PSCs is initiated by damaged acinar cells and reinforced by already activated PSCs in a paracrine and autocrine manner through synthesis and secretion of pro-fibrotic mediators. Thus, we investigated whether the reduced fibrosis observed upon MS-275 treatment was linked to reduced expression of these factors. In support of this hypothesis, gene expression levels of pro-fibrotic TGFβ1-3 were lower in the presence of the inhibitor (Fig. 4A). Consequent to the deceased production of TGFβ isoforms, activation of TGFβ signaling was hampered, as shown by reduced phosphorylation of Smad3, an initiating event of the intracellular cascade resulting upon engagement and dimerization of TGFβ receptor complex (Fig. 4B). Interestingly, TGFβ receptor II was also up-regulated following induction of pancreatitis, however its expression levels were not reduced following MS-275 treatment (Fig. 4C), thus suggesting that the inhibitor decreased TGFβ signaling by reducing the expression of TGFβ isoforms rather than lowering the expression of their receptors.

Inhibition of class I HDAC with MS-275 reduces TGFβ expression in pancreatic acinar cells.

Given the pivotal role played by TGFβ in the development of pancreatic fibrosis, we further evaluated the identity of TGFβ-producing cells that are targeted by MS-275. Previous works showed that TGFβ is synthesized in activated PSC, thus acting through autocrine loops (reviewed in (9)). In our study, however, we investigated whether the synthesis of TGFβ upstream of PSC activation is inhibited by MS-275. Specifically, we asked whether damaged acinar cells following induction of pancreatitis up-regulate TGFβ expression in a HDAC-dependent manner. To exclude the confounding element of inflammatory and stromal components activated and recruited to the pancreas *in vivo* upon cerulein administration, we isolated primary pancreatic acini (Fig. 5A) and treated them *in vitro* with the drug. Cerulein treatment increased the expression of HDACs in isolated acini, especially evident for the HDAC1 isoform (Fig. 5B), suggesting that up-regulation of these enzymes is independent from the presence of stromal cells. Importantly, cerulein treatment also up-regulated the expression of TGFβ in acinar cells and pre-treatment with MS-275 abrogated TGFβ induction (Fig. 5C), indicating that the inhibitor directly reduces TGFβ production in acinar cells.

DISCUSSION

A detailed understanding of the mechanisms driving the fibrotic process in the pancreas is still lacking and its achievement would constitute an essential component to design novel therapeutic intervention to cure this severe disease. TGFβ is a potent pro-fibrotic factor and plays a pivotal role in the development of fibrosis in chronic pancreatitis, as demonstrated by a conspicuous body of research in this field using a variety of genetic and pharmacological approaches to alter the levels of this molecule or inactivate its function (10-13). Importantly, these studies highlighted that fact that the TGFβ pathway is not only critical for the development of pancreatic fibrosis, but also has the potential to be targeted with beneficial therapeutic outcomes.

One of the main effects exerted by TGFβ is activation of PSCs, which consists in a transformation from a quiescent state to a myofibroblast-like phenotype (reviewed in (9)). In recent years, activated PSCs have attracted increasing attention as major mediators of pancreatic fibrosis during chronic pancreatitis, as they not only mediate the development of fibrosis by producing ECM proteins, but also amplify the fibrotic response in an autocrine and

paracrine manner by secreting pro-fibrotic factors, including TGFβ (14). In the present study we discovered that expression and activity of HDACs in the pancreas is functionally linked with the development of organ fibrosis during chronic pancreatitis, thus providing a potential therapeutic target to counteract this disease. This hypothesis was further tested in *in vivo* experiments where the selective inhibitor of class I HDACs MS-275 was administered in a preventive or therapeutic manner during chronic pancreatitis. We observed a striking inhibition of pancreatic fibrosis and improved parenchima preservation in both regimen types, suggesting that MS-275 exerts an anti-fibrotic effect even when administered after the commencement of the disease.

At the cellular level, the reduced fibrosis detected upon MS-275 treatment was likely the result of reduced TGFβ expression, leading to a reduced conversion of PSCs from a quiescent to an activated state. An important question arising from these data is the identity of the cells whose TGFβ production is targeted by the inhibitor. Using *in vitro* experiments with isolated acinar cells we found that short term incubation with cerulein was sufficient to stimulate TGFβ expression in these cells. The fact that MS-275 treatment potently reduced cerulein-stimulated TGFβ expression suggests that acinar cells are indeed a direct target of the inhibitor and contribute to the phenotype observed *in vivo*.

However, it is likely that acinar cells are not the unique source of TGFβ in the pancreas, as previous studies reported the presence of TGFβ1 mRNA in stromal cells upon induction of pancreatitis (15). In this regard it is important to mention that TGFβ synthesis in non-acinar cells may also depend on HDAC activity. In fact, treatment of isolated PSCs with the pan-HDAC inhibitor sodium valproate was reported to inhibit TGFβ expression and collagen synthesis in these cells (16). Furthermore, another possible source of TGFβ production are inflammatory cells, which are recruited to the pancreas during the development of pancreatitis. This is of particular interest as we recently demonstrated that MS-275 treatment effectively reduced the levels of inflammation during the course of acute and chronic pancreatitis (Manuscript A). Thus, while it is possible that reduced inflammation upon MS-275 administration leads to reduced fibrosis during chronic pancreatitis, a recent study revealed that development of inflammation and fibrosis are two independent and not causal events in this disease. Specifically, using transgenic mice deficient in Cxcr2, the authors observed almost complete ablation of inflammatory cell infiltration upon chronic pancreatitis. However, this limited inflammatory reaction did not prevent PSC activation and, consequently, fibrosis levels were comparable in transgenic and wild type control mice (17). This striking example implies that signaling molecules derived inflammatory cells, including TGFβ1, may play a minor role in the development of pancreatic fibrosis during chronic pancreatitis.

Collectively, our results integrate with the current body of evidences demonstrating the critical role of HDACs in the development of fibrotic diseases. Examples in this regard are compelling evidences that HDAC activity is necessary for activation of hepatic stellate cells *in vitro* (recently reviewed in (4)). The requirement of HDAC activity in driving myofibroblastic differentiation and ECM protein synthesis was also observed in different fibroblast populations present in skin, lung, kidney (18-20). Moreover, the use of different HDAC inhibitors showed beneficial effects in the treatment of hepatic, renal, cardiac, pulmonary fibrosis *in vivo* (4,21-27). This suggests that epigenetic mechanisms controlled by HDACs may be a conserved feature in the development of different fibrotic diseases.

Conclusion

Counteracting the development of pancreatic fibrosis is a major and still unachieved therapeutic goal in the context of chronic pancreatitis. Our data revealed a potent anti-fibrotic effect of MS-275

treatment, which is mediated at least in part by suppression of TGFβ expression in acinar cells. This suggests on one hand that class I HDAC activity is critical for the timely controlled epigenetic regulation of key signaling molecules driving the development of fibrosis in this organ. On the other hand, this provides a new perspective on the cell type involved in regulating the process and highlights the possibility that acinar cells act as active mediators of pancreatic fibrosis.

These results harbor important implications to further evaluate the therapeutic value MS-275 in the context of chronic pancreatitis patients. Yet, future investigations using conditional knocked out mouse models are needed to achieve a global understanding of the individual HDAC isoforms' function in the different cell types that are involved in the development of this disease.

ACKNOWLEDGMENTS

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Author Contributions

The authors of this manuscript contributed in the study design, acquisition, analysis, interpretation of data, and critical revision of the manuscript. MB performed experiments, generated and analyzed data, drafted the manuscript; ES, EM, RC performed experiments, generated and analyzed data; RG revised the manuscript; SS designed the study, wrote the manuscript. All authors approved the submitted version.

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FIGURE LEGENDS

Figure 1. Characterization of chronic pancreatitis development. (**A**) Haematoxylin and eosin (H&E) and (**B**) Masson's Trichrome staining of pancreata at the indicated weeks of pancreatitis induction revealed progressive destruction of parenchima morphology, cell infiltration and collagen deposition (green staining). (**C**) qPCR of collagen expression in the pancreas at the indicated weeks of pancreatitis. (**D**) qPCR of α-smooth muscle (α-Sma) expression in the pancreas at the indicated weeks of pancreatitis, indicative of pancreatic stellate cell activation. (**E**) qPCR of TGFβ isoforms and TGFβ receptor II (TGFβRII) expression in the pancreas at the indicated weeks of pancreatitis. (**F**) HDAC activity in pancreata in the pancreas after four week of pancreatitis.
Results are average ± SEM (n=5), **P* < 0.05. Scale bars: 50 μm.

Figure 2. Preventive and therapeutic administration of MS-275 reduces the development of fibrosis during chronic pancreatitis. (**A**) Schematic representation of two weeks of preventive (MS+Cer) and therapeutic (Cer+MS) MS-275 regimens during induction of chronic pancreatitis. Cerulein (Cer) was administered on alternate days over four weeks. MS-275 was administered on alternate days over two weeks. (**B**) Masson's Trichrome staining of pancreata showing reduced collagen deposition (green) after four weeks of chronic pancreatitis following preventive and therapeutic MS-275 regimens. (**C**) Densitometric quantification of collagen deposition visualized with Masson's Trichrome staining. Results are expressed as % of the total pancreatic area. (**D**) qPCR of collagen expression.
Results are average ± SEM (n=5), **P* < 0.05. Scale bars: 50 μm.

Figure 3. Preventive and therapeutic administration of MS-275 reduces the activation of pancreatic stellate cells during chronic pancreatitis. (**A**) qPCR of α-smooth muscle (α-SMA) expression in pancreata after four weeks of chronic pancreatitis following preventive and therapeutic MS-275 regimens. (**B**) Western blotting quantification of α-SMA in pancreata after four weeks of chronic pancreatitis following preventive and therapeutic MS-275 regimens. Band intensity values were normalized using tubulin as a loading control.
Results are average ± SEM (n=5), **P* < 0.05.

Figure 4. Preventive and therapeutic administration of MS-275 reduces the expression of TGFβ during chronic pancreatitis. (**A**) qPCR of TGFβ isoforms expression after four weeks of chronic pancreatitis following preventive and therapeutic MS-275 regimens. (**B**) Western blotting quantification of phospho-SMAD3 in pancreata after four weeks of chronic pancreatitis following preventive and therapeutic MS-275 regimens. Band intensity values were normalized using Gapdh as a loading control. (**C**) qPCR of TGFβ receptor II (TGFbRII) expression in pancreata after four weeks of chronic pancreatitis following preventive and therapeutic MS-275 regimens.
Results are average ± SEM (n=5), **P* < 0.05.

Figure 5. MS-275 administration inhibits TGFb expression in acinar cells. (**A**) Micrograph of isolated pancreatic acinar cells. (**B**) qPCR of class I HDAC expression in isolated acinar cells upon *in vitro* treatment with cerulein. (**C**) qPCR of TGFβ1 expression in isolated acinar cells upon *in vitro* treatment with cerulein in the presence or absence of MS-275.
Results are average ± SEM (n=5), **P* < 0.05. Scale bars: 50 μm.

Figures:

Figure 1

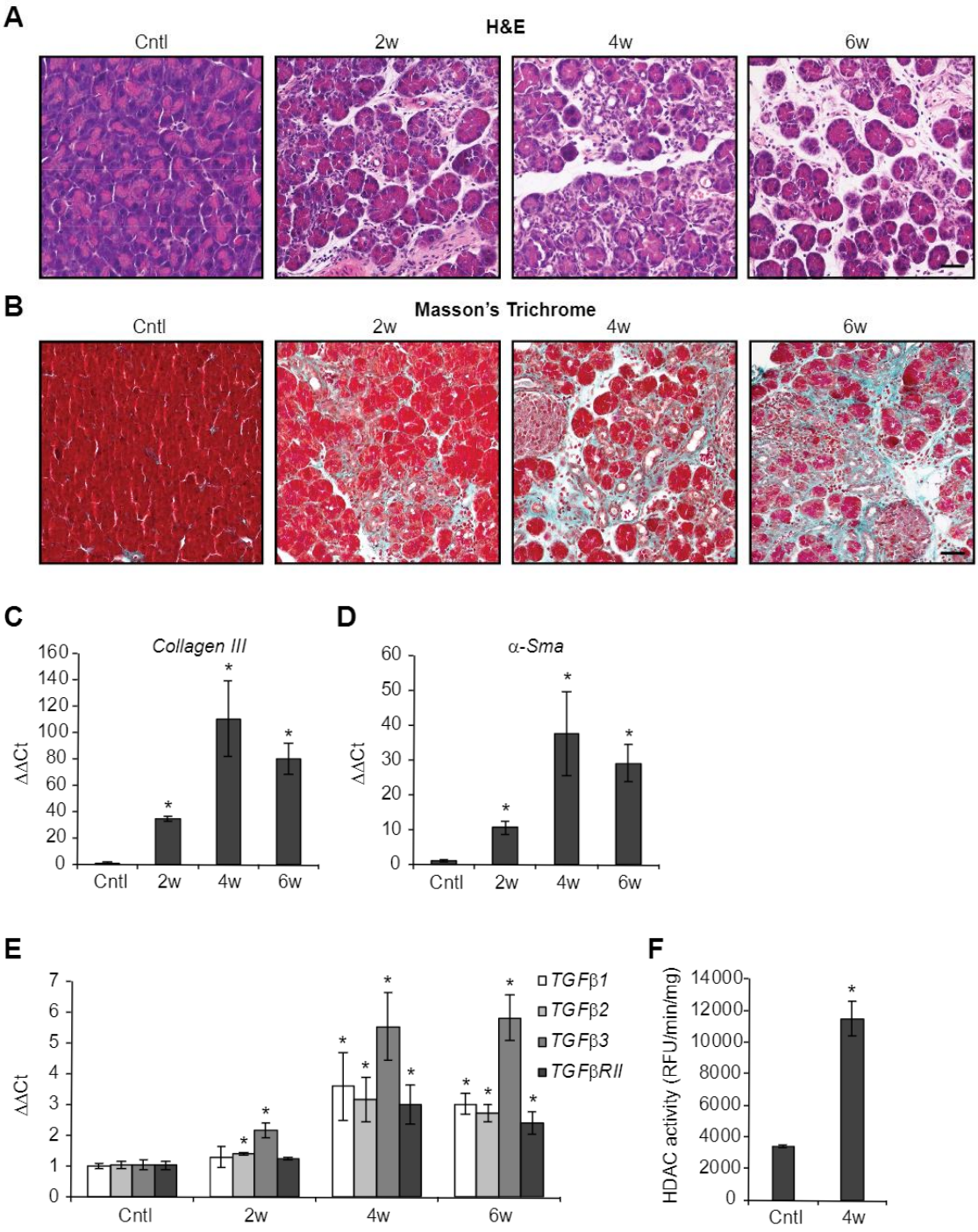
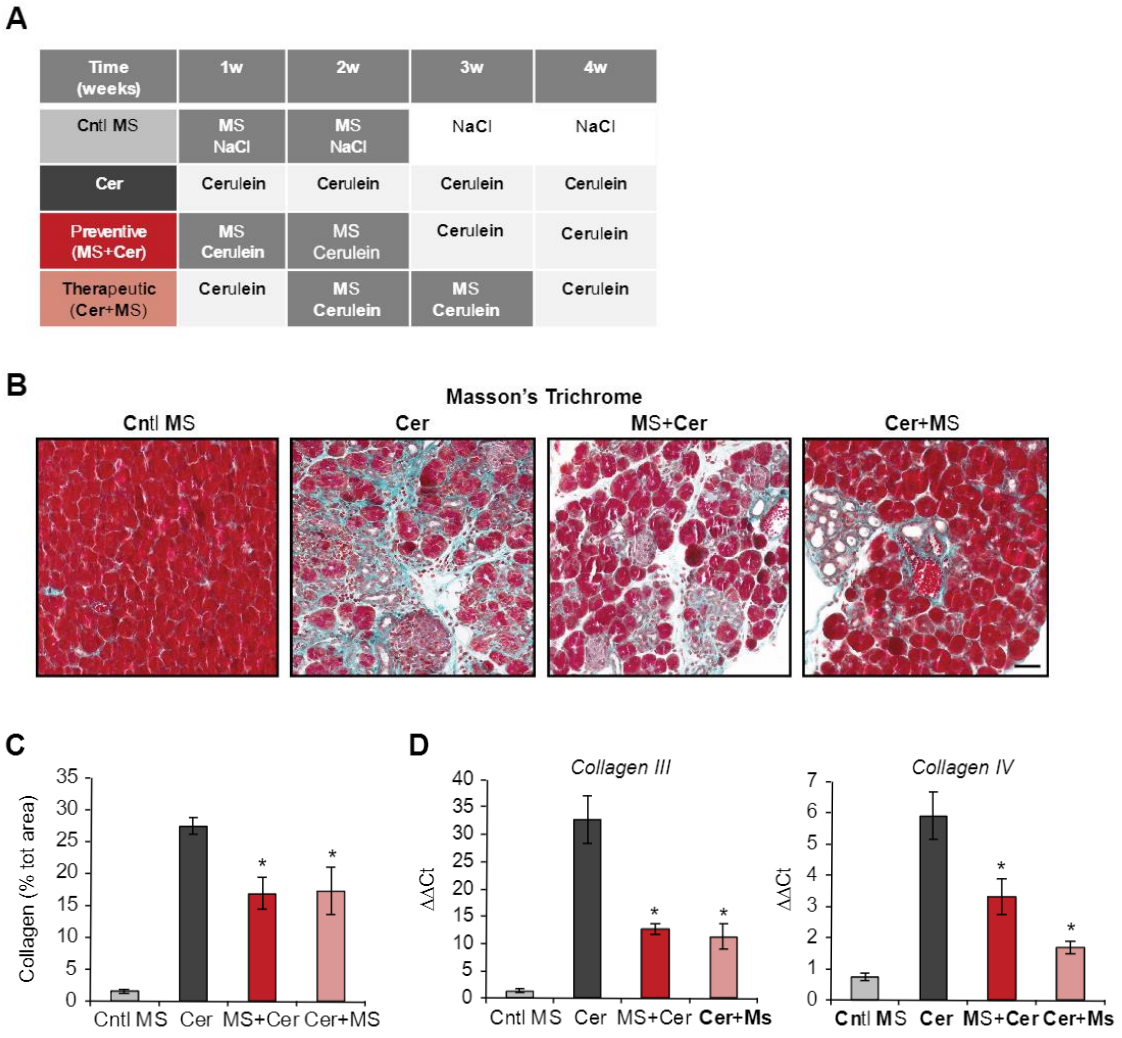


Figure 2



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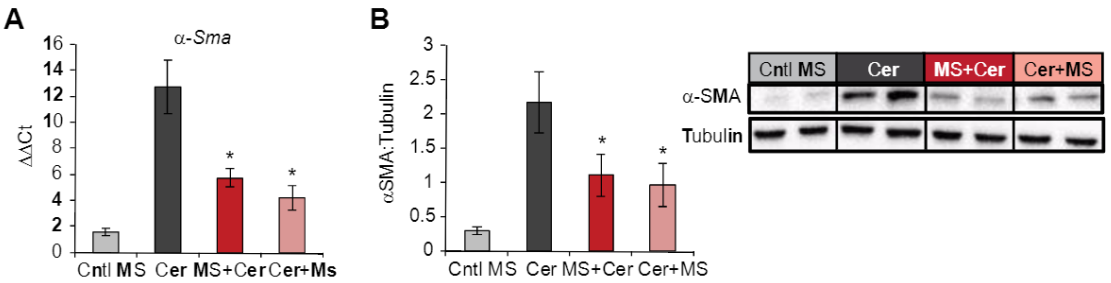


Figure 4

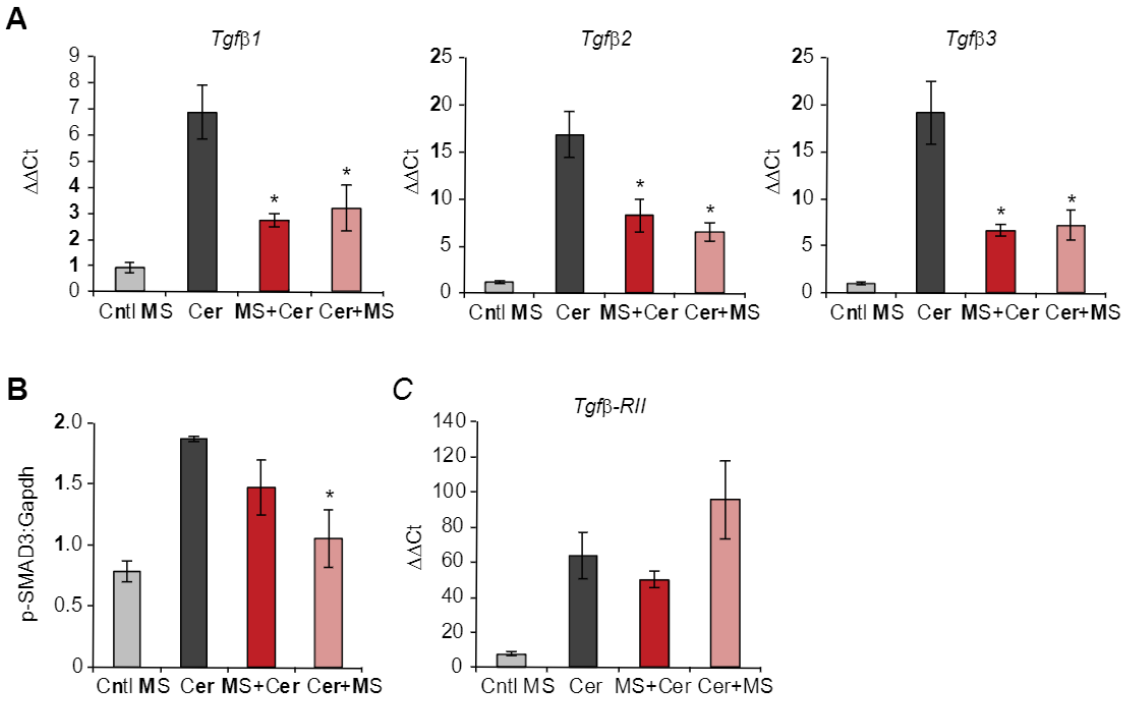
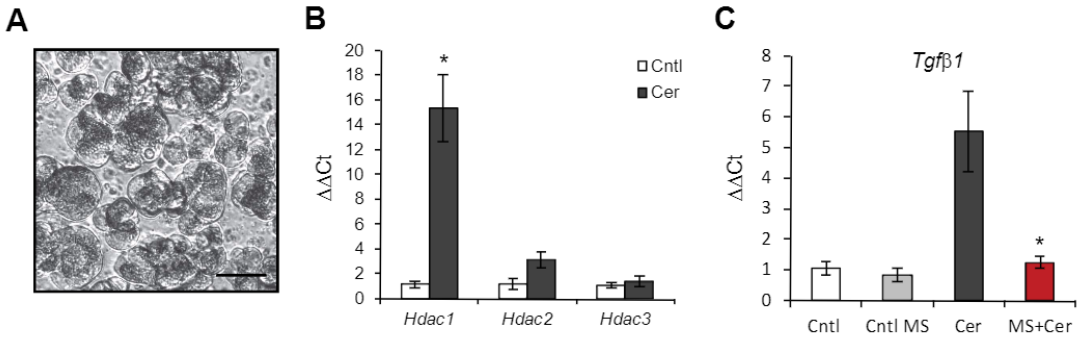


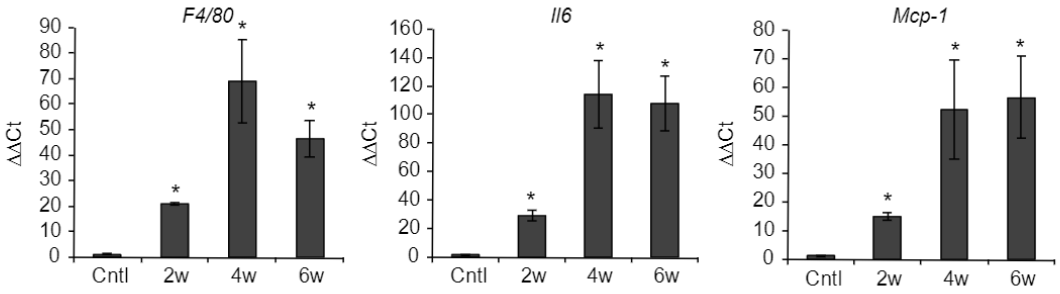
Figure 5



SUPPLEMENTARY FIGURE LEGENDS

Supplementary figure 1. qPCR of inflammatory markers in pancreata at the indicated weeks of pancreatitis induction. F4/80, marker of activated macrophages; IL6, interleukin 6; MCP-1, monocyte chemotactic protein 1. Results are average ± SEM (n=5), **P* < 0.05.

Figure S1



Discussion & Conclusions

6. Discussion

A combination of epigenetic modifications generate a “histone code”. This histone code is vital to regulate gene expression independently of DNA sequence. Acetylation, methylation, phosphorylation and ubiquitination are the epigenetic modifications that occur on specific residues of histone tails and induce alterations in the helix of DNA affecting protein-protein interactions [59, 60]. The discovery of epigenetic modifications that affect gene expression has added another layer of complexity to the already tightly controlled regulatory machinery of the epigenome to control cell function, regulation, and identity. Histone acetylation is one of the best-studied epigenetic mechanisms. A dynamic balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulates the status of histone acetylation. The balance between these enzymes is a key regulatory mechanism for gene expression and governs numerous developmental and disease state and cellular processes. Indeed, it has been demonstrated that elevated HDAC activity is required in embryonic development and organogenesis, while a dysregulation of HDACs has been linked to cancer, cardiovascular, immune and fibrotic disorders. Therefore, a big amount of basic research studies and human clinical trials are investigating the use of HDAC inhibitors as a treatment for a variety of disorders. Indeed, in this study we investigated the role of HDACs in the different phases that characterize the development of pancreatitis and the therapeutic value of HDAC inhibition.

6.1 HDACs in early acinar cell injury and pancreatic inflammation development.

Collectively, our data obtained from different models of pancreatitis provided the evidence that from the early onset to the chronic phase of pancreatitis, class I HDACs and nuclear HDAC activity were significantly increased. This was concomitant with a decrease in protein acetylation. Furthermore, HDAC up-regulation was restricted to pancreatic tissue, as HDAC expression did not change in other organs 24h after induction of pancreatitis.

Given the early HDACs up-regulation, specifically of class I HDAC, we investigated whether class I HDACs were promoting the onset of acinar cell damage. To assess the functional HDAC role during the initial phase of pancreatitis, we used MS-275, a selective inhibitor of class I HDAC. The initial acinar cell damage was measured by serum amylase, trypsinogen activation and acinar cell death. In any of our different mouse strains the parameters named above were not affected by class I HDAC inhibitor MS-275, injected one day before cerulein-induced pancreatitis. Contrary to what was previously published. The pretreatment with the pan-HDAC inhibitor trichostatin A, which targets class I and class II HDACs, reduced the initial pancreatic damage evoked by taurocholate-induced acute pancreatitis [56]. Thus, differently from pan-HDAC inhibitor trichostatin A, the administration of MS-275 does not prevent the initial acinar cell damage. This data suggests that different HDAC classes, and possibly individual class members, may have different and non-redundant functions depending on the kind of initial damage. However, the two models represent different severities of pancreatitis and thereby, HDAC function may be different. To resolve these apparently conflicting results, further studies using conditional KO mice for the different HDAC isoforms are needed to validate this concept. Moreover, an initial acinar cell damage triggers the recruitment of leukocytes in the pancreas helping to develop an inflammatory response. The previous study using a pan-HDAC inhibitor showed reduced transcript levels for chemokines necessary to recruit leukocytes [56]. However, expression of the chemokines CXCL2 and

MCP-1 were not reduced in the presence of MS-275 in our model of cerulein-induced acute pancreatitis. Thus, these data are suggesting that the signals for leukocyte recruitment do not depend on class I HDACs. Despite the unchanged acinar cell damage and chemokine expression by MS-275 treatment, we provide evidence how MS-275 potently reduces the development of inflammation in the pancreas. This indicates that MS-275 reduced pancreatic inflammation independently from the initial acinar cell damage. A hypothesis explaining the observation that leukocytes are not responding properly to the injury is that MS-275 may have a direct inhibitory effect on leukocyte sub-populations. In support of this hypothesis, we have demonstrated that both, in the acute and chronic settings of pancreatitis, the recruitment of M1 and M2 types of macrophages and CD3-positive T cells were reduced in the presence of MS-275. Macrophages are one of the most relevant cell type in pancreatitis as they orchestrate both initiation and resolution of pancreatic inflammation. Furthermore, our in-vitro analyses with Raw264.7 macrophages showed that activation of these cells with the pro-inflammatory stimulus lipopolysaccharide (LPS) resulted in increased expression of HDAC1 and increased nuclear HDAC activity. Importantly, incubation of Raw264.7 macrophages with MS-275 reduced their HDAC activity and altered their cytokine production. Together, these data suggest that MS-275 has a direct effect on macrophage activation. Interestingly, MS-275 treatment did not homogeneously repress cytokine expression in macrophages, but rather de-regulate their transcription in a selective manner. Specifically, IL-6 was strongly down-regulated, while IL1β was up-regulated in both, RAW264.7 and primary isolated macrophages upon LPS activation. The biological implications of differential cytokine expression following MS-275 treatment remains to be further elucidated, as they are able to exert both pro-inflammatory and anti-inflammatory functions. An example in this regard is the finding that up-regulation of IL1β can induce apoptosis of bacterial-infected macrophages by inducing TNFα [61]. Thus, up-regulation of this cytokine observed in activated macrophages after MS-275 treatment suggested that they might promote macrophage apoptosis and consequently reduced infiltration of these cells in the pancreas during pancreatitis. Taken together, these results suggest that not only different leukocyte sub-populations are responsive to class I HDAC inhibition, but also that the molecular signals to develop pancreatic inflammation depends on class I HDAC activity. In fact, MS-275 treatment was previously shown to reduce the inflammatory response in animal models of rheumatoid arthritis [49], prostatitis [62], and neuritis [63]. Furthermore, treatment with other pan-HDAC inhibitors also ameliorated a wide spectrum of inflammatory diseases [64], highlighting the potential of targeting HDAC enzymes as a therapeutic strategy. Moreover, with our results showing a robust decrease of leukocyte recruitment following MS-275 treatment during the course of acute and chronic pancreatitis, the possible application of MS-275 as anti-inflammatory agents may be expanded to pancreatitis. However, caution is warranted when using pan-HDAC inhibitors for clinical applications. A major issue with HDAC inhibitors is that HDAC isoforms may have different and non-redundant effects in the development of inflammatory diseases. In addition, many targets of HDAC inhibitors are currently not known Thus, there is much to learn about how HDAC inhibitors affect gene expression. However, it is clear that in the case of pancreatitis, the class I HDAC inhibitor MS-275 is predicted to be efficacious. Since the acetylated modification represents a novel anti-inflammatory strategy through regulating the expressions of inflammatory genes, characterization of the role of individual HDACs in driving inflammatory responses

may ultimately lead to the development of more selective HDAC inhibitors for specific anti-inflammatory applications depending on species, cell type, or the specific inflammatory mediators [65].

6.2 HDACs in pancreatic regeneration

The pancreas has a limited ability to recover and regenerate after injury. The pancreatic regenerative process is characterized by a transient de-differentiation of acinar cells to acinar-to-ductal metaplasia (ADM) [20, 25]. It is a complex reprogramming event observed during the regenerative phase of pancreatitis and is characterized by a cellular phenotype that expresses markers for pancreatic progenitor cells such as Sox9. Since aspects of embryonic development of the pancreas are re-capitulated during recovery and regeneration following pancreatitis, it seems possible that the reactivation of progenitor genes during pancreatitis are regulated in a similar manner by HDAC activity following pancreatitis. Indeed, our results showed that acinar de-differentiation into ADM also was accompanied by increased HDAC expression. On the other hand, MS-275 treatment was sufficient to reduce HDAC expression and activity preventing ADM formation both, *in vitro* and *in vivo.* Previously, we described the correlation between inflammation levels and HDAC expression and it is know that macrophages can trigger ADM. Thus, ADM reduction seems to be an indirect effect derived from the reduction of macrophage infiltration after MS-275 treatment. However, we observed up-regulation of HDAC expression in the isolated acinar cells in the absence of inflammatory cells. This result corroborated the concept that class I HDACs activity was increased in acinar cells following induction of pancreatitis. Furthermore, it was necessary to induce acinar cell de-differentiation into ADM by cell-autonomous mechanisms. However, reduced ADM formation upon MS-275 treatment may result from other processes beside this specific epigenetic reaction. In fact, our results highlighted EGF receptor (EGFR) signaling pathway as underlying molecular mechanism targeted by class I HDAC inhibition. We observed a reduction of ADM by MS-275 concomitant with reduced expression of EGFR in both *in-vivo* and *in-vitro* systems reducing acinar de-differentiation. These findings support the essential function of HDACs for acinar de-differentiation during development and in the adult state following inflammatory insult. It appears that EGFR signaling is part of the underliying molecular mechanism targeted by class I HDAC. Importantly, the reactivation of developmental genes following pancreatitis culminates in an increased acinar cell proliferation. Class I HDACs have been found to play a central role in controlling cell cycle regulation including proliferation in non-cancerogenic mammalian systems [66]. Our mouse model showed reduced number of proliferating acinar cells after MS-275 treatment in acute and chronic pancreatitis. This phenotype could result from a reduced DNA damage in acinar cells, reduced inflammation and reduced ADM. Nevertheless, we cannot exclude a direct effect of MS-275 on acinar cell cycle progression. Indeed, our results indicated that decreased replication of acinar cells observed following MS-275 treatment was due to a decreased expression of late cyclins. In addition, more recently the same effect have been observed in other non-transformed cells, e.g. in the mouse liver after partial hepatectomy (PH). Following PH, the hepatic class I HDAC activity was significantly increased in nuclear and cytoplasmic fractions. Furthermore, these changes in hepatic class I HDAC expression, subcellular localization, and activity was concomitant with diminished histone acetylation during the regenerating liver phase [42]. Interestingly, when these mice were treated with suberoylanilide hydroxyamic acid (SAHA, a pan-HDAC inhibitor) an anti-regenerative effect was observed. SAHA

induced a delay on PH-induced histone deacetylation, damped hepatocellular proliferation, and blunted hepatic expression and activation of cell cycle signals downstream of cyclin D1 expression. Thus, this study demonstrates that HDAC activity promotes liver regeneration by regulating hepatocellular cell cycle progression downstream of cyclin D1 induction [42]. Overall, our data demonstrate that inhibition of class I HDAC with MS-275 ameliorates the outcome of acute pancreatitis. However, care has to be applied in the choice of the HDAC inhibitor. For example valproic acid treatment, an antiepileptic drug that also showed a pan-HDACs inhibitory activity, was associated with development of pancreatitis and multiorgan failure in humans [67-69]. In mice, it caused disease exacerbation, manifested by increased inflammation, decreased proliferation and persistent acinar de-differentiation with non-resolving ADMs [53]. At this point it is important to mention that prolonged de-differentiation of acinar cells into ADMs is unfavorable for pancreatic recovery following pancreatitis. Moreover, ADMs are considered premalignant lesions associated with the risk to develop pancreatic cancer. Therefore, further experiments are needed to assess the specific role of HDACs isoforms in non-transformed cells.

6.3 HDACs in the development of pancreatic fibrosis

The pathogenesis of chronic pancreatitis is characterized by development of pancreatic fibrosis. Pancreatic fibrosis impairs exocrine and endocrine functions of the pancreas, leading to pancreatic dysfunction and severe impairment of patient's quality of life. In pancreatic fibrosis the equilibrium of extracellular matrix (ECM) formation and degradation is impaired, resulting in excessive deposition of ECM by an activated population of pancreatic stellate cells (PSCs). These activated PSCs transdifferentiate into myofibroblasts. They have characteristics that are intermediate between fibroblasts and smooth muscle cells, express alpha smooth muscle actin (α-SMA) and contractile proteins. In addition, they have higher rates of cellular proliferation and extracellular matrix deposition compared to fibroblasts. Therefore, they are considered the main producers of ECM, when become activated in the inflamed pancreas. ECM production in PSCs is regulated by fibrogenic growth factors. The main growth factors are transforming growth factor β1 (TGF-β1), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), among other cytokines. These are produced by damaged acinar cells, inflammatory cells and activated PSCs. Thus, they can act in a paracrine and autocrine manner on PSCs. In particular, TGF-β is a key regulator of ECM formation and remodeling. It induces the expression of ECM proteins, proteases, and their inhibitors, such as the tissue inhibitors of metalloproteinases (TIMPs). Our *in vivo* data revealed that MS-275, in both preventive and therapeutic regimens, limited the activation of PSCs and reduced collagen deposition following induction of pancreatitis. These data complement previous reports showing that HDAC inhibitors reduced the activation of PSCs and ECM deposition *in vitro* [70] and reduced the expression of pro-fibrogenic factors in these cells, suggesting that HDAC inhibitors may inhibit the autocrine activation of PSCs. Interestingly, we found that MS-275 directly inhibited TGF-β expression in explanted acinar cells following cerulein-induced damage. Thus, the capacity of MS-275 to reduce the production of TGFβ in acinar cells, result in a diminished paracrine activation of PSC and consequent ECM deposition. These findings imply that MS-275 not only exerts a therapeutic effect in the early steps of the development of fibrosis upon induction of chronic pancreatitis, but also that it reduces pancreatic fibrosis by targeting both PSC and acinar cells. In addition to our findings, another study using a general pan-HDAC inhibitor, sodium

butyrate, demonstrated that HDACi are potent anti-fibrotic drugs in a different model of chronic pancreatitis. Specifically, the authors of this study showed that sodium butyrate treatment ameliorated the severe histological pancreatic changes observed during L-Arginine induced chronic pancreatitis in Wistar rats, [54]. Similar to our results using MS-275, sodium butyrate reduced collagen deposition and PSC activation, with consequent decrease of pancreatic fibrosis. Based on these similar results obtained with pan-HDAC and selective class I HDAC inhibitors it is likely that class I HDACs are the isoforms mainly responsible for the development of pancreatic fibrosis, however further studies are necessary to confirm this hypothesis.

The anti-fibrotic effect of MS-275 is not restricted to the pancreas but has also been reported in the development of renal fibrosis [52]. In this study the authors observed less tubular dilatation, ECM deposition and fibroblast activation in the mouse model of unilateral ureteral obstruction (UUO) after MS-275 treatment compared to untreated kidney. Furthermore, the authors also demonstrated that the molecular mechanism behind reduced fibrosis following MS-275 treatment is the inhibition of TGB-β expression [52]. Similar anti-fibrotic effects have been shown for pan-HDAC inhibitors in the case of skin and liver fibrosis [71-73].

One important point that emerges from the available literature is that most of the studies focus on the effect of HDAC inhibitors, but the contribution of the individual HDAC isoforms during fibrosis is far from understood. Elucidating the role of HDAC isoforms would be highly relevant in clinical settings as: 1) different isoforms may have opposing roles in the development of fibrosis, 2) identifying the isoforms with pro-fibrotic roles would facilitate the development of specific inhibitors, which will likely result in more effective therapies than using pan-HDAC inhibitors. An example in this regard is the characterization of the functional role of different HDAC isoforms in cardiac fibrosis, which represents an adoptive response in the heart exposed to various stress (reviewed in [38]). These studies showed that class I and IIa HDACs have opposing functions in the regulation of fibrosis. Indeed, by use of HDAC2 knockout or transgenic mice investigating the function of cardiomyocytes, it turns out that HDAC2, a class I HDAC, induces cardiac hypertrophy in response to early hypertrophic stimuli whereas, class IIa HDACs including HDAC4, HDAC5, and HDAC9 suppress cardiac hypertrophy [38]. Thus, more research should be done using KO mice to reveal the specific mechanism by which individual HDACs participate in fibrosis.

6.4 Conclusions

The study reported here demonstrated that HDAC activity and expression were up-regulated in the pancreas during acute, chronic and autoimmune pancreatitis in different mouse models. In addition, we demonstrated the therapeutic value of the class I HDAC inhibitor Ms275, which can ameliorate the outcome of both acute and chronic forms of pancreatitis by reducing leukocyte recruitment, acinar-to-ductal metaplasia and fibrosis. Furthermore, our data directly link the activity of class I HDACs with the pathophysiology of this severe disease. The involvement of these enzymes in the development of the inflammatory response, the de-differentiation of acinar cells and fibrosis production during acute and chornic pancreatitis, and their therapeutic value is summarized in Figure 4. Up-regulation of HDAC isoforms observed during autoimmune pancreatitis prompts the exploration of the effect of HDAC inhibition in this type of chronic pancreatitis for which the treatment remains challenging due to the high

frequency of relapses. While the safety of several HDAC inhibitors as anti-cancer treatments has been ascertained, including MS-275 [74], additional clinical trials are needed to determine the correct therapeutic regimen in the setting of pancreatitis. In addition, given the existence of many HDAC isoforms, further studies are required to understand their specificity or redundancy of functions, based on which isoform-selective inhibitors can be exploited to minimize toxicity.

Figure 4.

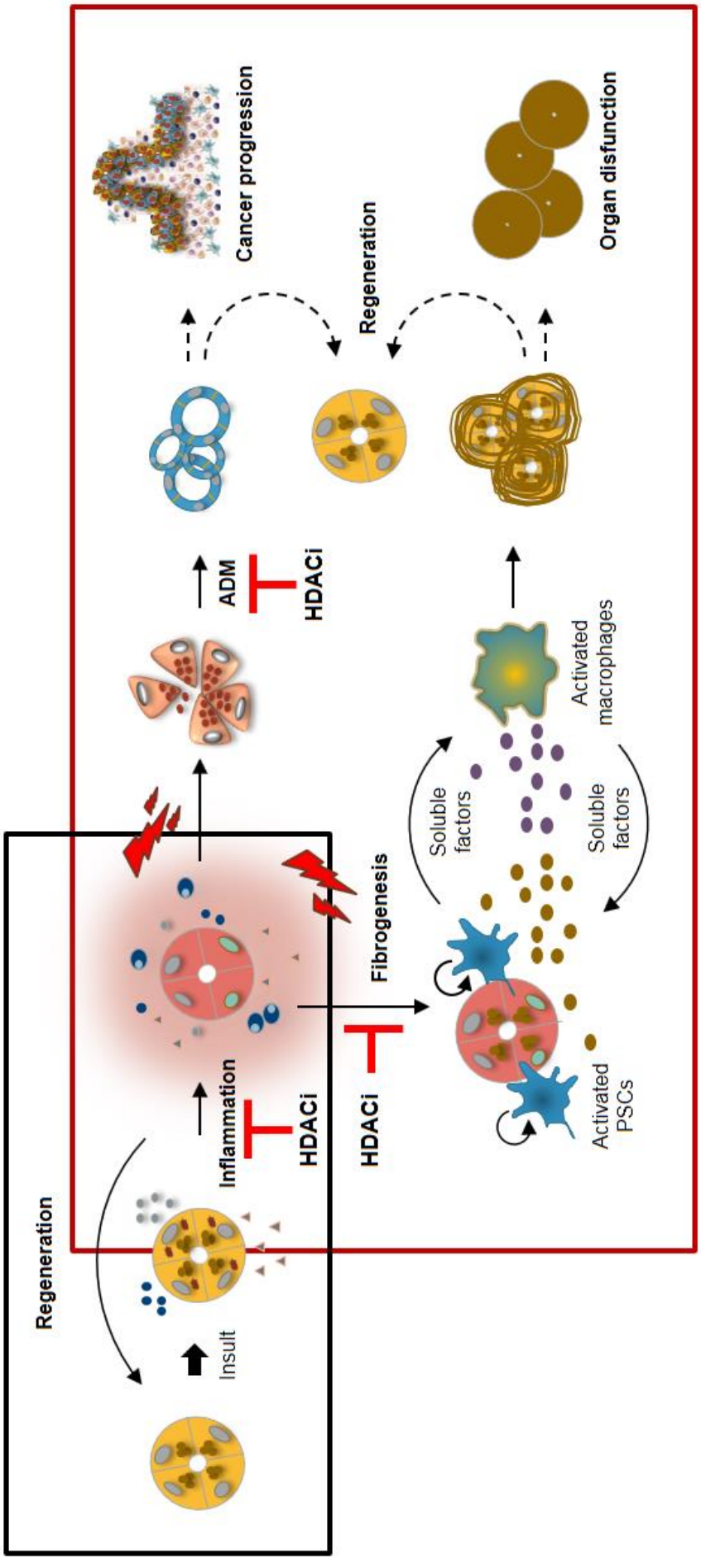


Figure 3. Schematic representation of pancreatitis pathophysiology evolution from the initial inflammatory response during AP and the following de-differentiation of acinar cells and fibrosis formation during CP. Furthermore, in red (arrow) indicate where HDACi have an effect during pancreatitis. Black square AP. Red square AP toward to CP. Solid lines, direct effects; dashed lines, potential outcomes of pancreatitis.

Curriculum vitae

Marta Bombardó Ayats

7. Curriculum vitae

Last name:	BOMBARDÓ AYATS
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Date of Birth:	04 March 1987
Nationality:	Spanish

Education

2013-present — Doctoral studies at Zurich Center for Integrative Human Physiology (ZIHP), PhD program in Integrative Molecular Medicine (ImMed), University of Zurich, Switzerland.
PhD thesis title: Inhibition of Class I HDAC ameliorates acute and chronic pancreatitis by reducing leukocyte recruitment, acinar-to-ductal metaplasia and fibrosis.
PI: prof. Dr. Rolf Graf and PD. Dr. Sabrina Sonda.

2011-2012 — International Master's Degree in Neurosciences, Faculty of Biology; University of Barcelona, Spain.
Master thesis: Deeper insight at cannabinoid receptor CB1 and CB2 in mouse pancreatic islets by the use of knockout models.
PI: prof. Rafael Maldonado and Dr. Blanca Rubí

2007-2011 — Second cycle of Human Biomedicine, Faculty of Health and life Sciences; Pompeu Fabra University, Spain.
Degree thesis: Identification of Cannabinoid Receptor I & II (CB1, CB2) in pancreatic islets.
PI: prof. Rafael Maldonado and Dr. Blanca Rubí

2005-2007 — First cycle of Biology; Campus Montilivi, University of Girona, Spain.

Additional training

2016
- BioBusiness Summer School (Amsterdam).
- Discovering Management at ETH Zurich.

2015
- The successful start of a professional career at UZH Zurich.
- Resources-focused stress management at UZH Zurich.

2014
- Scientific writing in the Sciences and Medicine at UZH Zurich.
- Introduction to human physiology: regulation of cardiovascular function at UZH Zurich.

2013

- Introduction to clinical research methods at Unil Lausanne.
- Méthodes en recherche clinique at Unil Lausanne.
- Perception du risque et santé at Unil Lausanne.
- Effects of sugar in the health (Sucré et santé) at Unil Lausanne.
- La nutrition sous une perspective génomique at Unil Lausanne.
- Mini-symposium Novel concepts in the metabolic syndrome: Connecting inflammation and metabolism at Unil Lausanne.
- Mini-symposium Lipotoxicity from the multi-organ perspective at Unil Lausanne.

Academic publication

2015 Saponara E, Grabliauskaite K, **Bombardo M**, Buzzi R, Silva AB, Malagola E, Tian Y, Hehl AB, Schraner EM, Seleznik GM, Zabel A, Reding T, Sonda S, Graf R. *"**Serotonin promotes acinar dedifferentiation following pancreatitis-induced regeneration in the adult pancreas**".* J Pathol, 2015. **237**(4): p. 495-507.

2016 *Grabliauskaite K, Saponara E, Reding T, **Bombardo M**, Seleznik GM, Malagola E, Zabel A, Faso C, Sonda S, Graf R. "**Inactivation of TGFbeta receptor II signalling in pancreatic epithelial cells promotes acinar cell proliferation, acinar-to-ductal metaplasia and fibrosis during pancreatitis**".* J Pathol, 2016. **238**(3): p. 434-45.

Submitted manuscripts

2016 **Marta Bombardo**, Enrica Saponara, Ermanno Malagola, Rong Chen, Gitta M. Seleznik, Cecile Haumaitre, Evans Quilichini, Anja Zabel, Theresia Reding, Rolf Graf and Sabrina Sonda. ***"Class I HDAC inhibition improves pancreatitis outcome by limiting leukocyte recruitment and acinar-to-ductal metaplasia"*** (Second submission) British Journal of Pharmacology.

Marta Bombardo, Ermanno Malagola, Rong Chen, Alina Rudnicka, Rolf Graf and Sabrina Sonda. ***"Ibuprofen treatment reduces the proliferation of pancreatic acinar cells upon inflammatory injury and mitogenic stimulation"*** (Second (Second submission) British Journal of Pharmacology.

2017 **Marta Bombardo**, Enrica Saponara, Ermanno Malagola, Rong Chen, Rolf Graf and Sabrina Sonda. ***"Inhibition of class I histone deacetylases abrogates TGFβ expression, pancreatic stellate cell activation and fibrosis during chronic pancreatitis "*** To be submitted in 2017.

Marta Bombardo, Ermanno Malagola, Rong Chen, Archangelo Carta, Gitta M. Seleznik, Rolf Graf and Sabrina Sonda. ***"Enhanced inflammatory responses in MRL/Mpj mice after pancreatic acinar cell injury"*** To be submitted in 2017.

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Prof. Dr. Jan Krützfeldt

Dipl. phil. Theresia Reding Graf

Dr. Gitta Seleznik, PhD

Ermanno Malagola

Rong Chen

Dr. Anurag Gupta, PhD

Sabrina Steiner

Ferran Sala

Núria Bombardó Ayats

Jaume Bombardó & Maite Ayats

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